



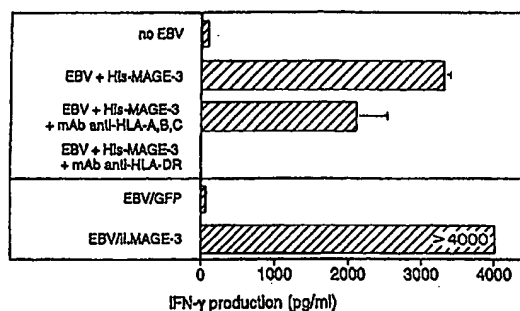
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : <b>C12N 15/12, C07K 14/47, G01N 33/574, C07K 16/30, C12N 5/06</b>		<b>A1</b>	(11) International Publication Number: <b>WO 00/20581</b>
			(43) International Publication Date: 13 April 2000 (13.04.00)
(21) International Application Number: PCT/US99/21230		(74) Agent: VAN AMSTERDAM, John, R.; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210 (US).	
(22) International Filing Date: 15 September 1999 (15.09.99)		(81) Designated States: AU, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: 09/166,448 5 October 1998 (05.10.98) US		Published With international search report.	
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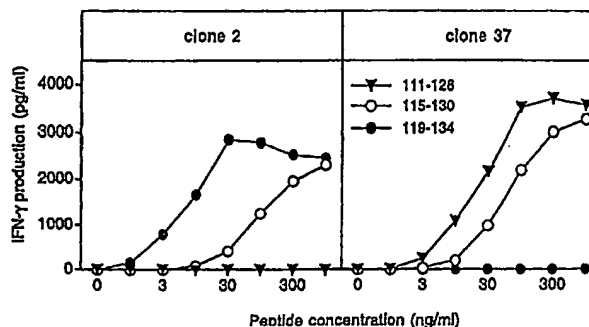
(54) Title: MAGE-A3 PEPTIDES PRESENTED BY HLA CLASS II MOLECULES

## (57) Abstract

The invention describes HLA class II binding peptides encoded by the MAGE-A3 tumor associated gene, as well as nucleic acids encoding such peptides and antibodies relating thereto. The peptides stimulate the activity and proliferation of CD4<sup>+</sup>T lymphocytes. Methods and products also are provided for diagnosing and treating conditions characterized by expression of the MAGE-A3 gene.



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**MAGE-A3 PEPTIDES PRESENTED BY HLA CLASS II MOLECULES****Field of the Invention**

This invention relates to fragments of the tumor associated gene product MAGE-A3  
5 which bind to and are presented to T lymphocytes by HLA class II molecules. The peptides,  
nucleic acid molecules which code for such peptides, as well as related antibodies and CD4<sup>+</sup> T  
lymphocytes, are useful, *inter alia*, in diagnostic and therapeutic contexts.

**Background of the Invention**

10 The process by which the mammalian immune system recognizes and reacts to foreign  
or alien materials is complex. An important facet of the system is the T cell response, which  
in part comprises mature T lymphocytes which are positive for either CD4 or CD8 cell surface  
proteins. T cells can recognize and interact with other cells via cell surface complexes on the  
other cells of peptides and molecules referred to as human leukocyte antigens ("HLAs") or  
15 major histocompatibility complexes ("MHCs"). The peptides are derived from larger  
molecules which are processed by the cells which also present the HLA/MHC molecule. See  
Male et al., Advanced Immunology (J.P. Lipincott Company, 1987), especially chapters 6-10.  
The interaction of T cells and complexes of HLA/peptide is restricted, requiring a specific T  
cell for a specific complex of an HLA molecule and a peptide. If a specific T cell is not  
20 present, there is no T cell response even if its partner complex is present. Similarly, there is  
no response if the specific complex is absent, but the T cell is present. The mechanisms  
described above are involved in the immune system's response to foreign materials, in  
autoimmune pathologies, and in responses to cellular abnormalities.

The T cell response to foreign antigens includes both cytolytic T lymphocytes and  
25 helper T lymphocytes. CD8<sup>+</sup> cytotoxic or cytolytic T cells (CTLs) are T cells which, when  
activated, lyse cells that present the appropriate antigen presented by HLA class I molecules.  
CD4<sup>+</sup> T helper cells are T cells which secrete cytokines to stimulate macrophages and antigen-  
producing B cells which present the appropriate antigen by HLA class II molecules on their  
surface.

30 The mechanism by which T cells recognize alien materials also has been implicated in  
cancer. A number of cytolytic T lymphocyte (CTL) clones directed against autologous  
melanoma have been described. In some instances, the antigens recognized by these clones

have been characterized. In De Plaen et al., *Immunogenetics* 40:360-369 (1994), the "MAGE" family, a family of genes encoding tumor specific antigens, is described. (See also PCT application PCT/US92/04354, published on November 26, 1992.) The expression products of these genes are processed into peptides which, in turn, are expressed on cell surfaces. This can lead to lysis of the tumor cells by specific CTLs. The genes are said to code for "tumor rejection antigen precursors" or "TRAP" molecules, and the peptides derived therefrom are referred to as "tumor rejection antigens" or "TRAs". See Traversari et al., *Immunogenetics* 35: 145 (1992); van der Bruggen et al., *Science* 254: 1643 (1991), for further information on this family of genes. Also, see U.S. Patent No. 5,342,774.

In U.S. Patent 5,405,940, MAGE nonapeptides are taught which are presented by the HLA-A1 molecule. Given the known specificity of particular peptides for particular HLA molecules, one should expect a particular peptide to bind one HLA molecule, but not others. This is important, because different individuals possess different HLA phenotypes. As a result, while identification of a particular peptide as being a partner for a specific HLA molecule has diagnostic and therapeutic ramifications, these are only relevant for individuals with that particular HLA phenotype. There is a need for further work in the area, because cellular abnormalities are not restricted to one particular HLA phenotype, and targeted therapy requires some knowledge of the phenotype of the abnormal cells at issue.

In U.S. Patent 5,591,430, additional isolated MAGE-A3 peptides are taught which are presented by the HLA-A2 molecule. Therefore, a given TRAP can yield a plurality of TRAs.

The foregoing references describe isolation and/or characterization of tumor rejection antigens which are presented by HLA class I molecules. These TRAs can induce activation and proliferation of CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) which recognize tumor cells that express the tumor associated genes (e.g. MAGE genes) which encode the TRAs.

The importance of CD4<sup>+</sup> T lymphocytes (helper T cells) in antitumor immunity has been demonstrated in animal models in which these cells not only serve cooperative and effector functions, but are also critical in maintaining immune memory (reviewed by Topalian, *Curr. Opin. Immunol.* 6:741-745, 1994). Moreover, several studies support the contention that poor tumor-specific immunity is due to inadequate activation of T helper cells.

It has recently been demonstrated that the tyrosinase gene encodes peptides which are presented by HLA class II molecules to stimulate CD4<sup>+</sup> T lymphocytes (Topalian et al., 1994; Yee et al., *J. Immunol.* 157:4079-4086, 1996; Topalian et al., *J. Exp. Med.* 183:1965-1971,

1996).

As with many cancer associated antigens, tyrosinase is expressed in a limited percentage of tumors and in limited types of tumors. Furthermore, the two identified MHC class II binding tyrosinase peptides are HLA-DRB1\*0401-restricted peptides, recognized only  
5 by cells which express the particular HLA molecule.

Therefore, there exist many patients who would not benefit from any therapy which includes helper T cell stimulation via tyrosinase peptides, either because the patient's tumor does not express tyrosinase, or because the patient does not express the appropriate HLA molecule. Accordingly, there is a need for the identification of additional tumor associated  
10 antigens which contain epitopes presented by MHC class II molecules and recognized by CD4+ lymphocytes.

### **Summary of the Invention**

It now has been discovered that the MAGE-A3 gene encodes additional tumor  
15 rejection antigens which are HLA class II binding peptides. These peptides, when presented by an antigen presenting cell having an HLA class II molecule, effectively induce the activation and proliferation of CD4<sup>+</sup> T lymphocytes.

The invention provides isolated MAGE-A3 peptides which bind HLA class II molecules, and functional variants of such peptides, the functional variants comprising one or  
20 more amino acid additions, substitutions or deletions to the MAGE-A3 peptide sequence.—The invention also provides isolated nucleic acid molecules encoding such peptides, expression vectors containing those nucleic acid molecules, host cells transfected with those nucleic acid molecules, and antibodies to those peptides and complexes of the peptides and HLA class II antigen presenting molecules. T lymphocytes which recognize complexes of the peptides and  
25 HLA class II antigen presenting molecules are also provided. Kits and vaccine compositions containing the foregoing molecules additionally are provided. The foregoing can be used in the diagnosis or treatment of conditions characterized by the expression of MAGE-A3. As it is known that the members of the MAGE family of polypeptides and nucleic acids share significant sequence identity and functional homology (e.g., as tumor antigens and  
30 precursors), the invention also embraces HLA binding peptides derived from members of the MAGE family other than MAGE-A3. Therefore, it is understood that the disclosure contained herein of MAGE-A3 HLA class II binding peptides, compositions containing such peptides,

and methods of identifying and using such peptides applies also to other members of the MAGE tumor associated antigen family.

According to one aspect of the invention, an isolated MAGE-A3 HLA class II-binding peptide, comprising a fragment of the amino acid sequence of SEQ ID NO:2 which binds an HLA class II molecule, or a functional variant thereof comprising one or more amino acid additions, substitutions or deletions, is provided. The isolated peptide in one embodiment comprises the amino acid sequence of SEQ ID NO:41, SEQ ID NO:42 or SEQ ID NO:86, or a functional variant thereof. In certain embodiments, the isolated HLA class II-binding peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:83 and SEQ ID NO:84. In preferred embodiments, the isolated peptide consists of one of the foregoing amino acid sequences. More preferably, the isolated peptide consists of an amino acid sequence selected from SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:86. In certain embodiments, the isolated peptide comprises an endosomal targeting signal, preferably an endosomal targeting portion of human invariant chain Ii. In other embodiments of the invention, the isolated HLA class II-binding peptide is non-hydrolyzable. Preferred non-hydrolyzable peptides are selected from the group consisting of peptides comprising D-amino acids, peptides comprising a -psi[CH<sub>2</sub>NH]-reduced amide peptide bond, peptides comprising a -psi[COCH<sub>2</sub>]-ketomethylene peptide bond, peptides comprising a -psi[CH(CN)NH]-(cyanomethylene)amino peptide bond, peptides comprising a -psi[CH<sub>2</sub>CH(OH)]-hydroxyethylene peptide bond, peptides comprising a -psi[CH<sub>2</sub>O]-peptide bond, and peptides comprising a -psi[CH<sub>2</sub>S]-thiomethylene peptide bond.

According to another aspect of the invention, a composition comprising an isolated MAGE-A3 HLA class I-binding peptide and an isolated MAGE-A3 HLA class II-binding peptide is provided. In certain embodiments, the MAGE-A3 HLA class I-binding peptide and the MAGE-A3 HLA class II-binding peptide are combined as a polytope polypeptide. In other embodiments the isolated MAGE-A3 HLA class II-binding peptide in the composition comprises the amino acid sequence of SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:86 or a functional variant thereof. Preferably, the isolated MAGE-A3 HLA class II-binding peptide in the composition consists of an amino acid sequence selected from the group consisting of

SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86. More  
5 preferably, the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:86. In certain embodiments of the foregoing compositions, the isolated MAGE-A3 HLA class II-binding peptide includes an endosomal targeting signal. Preferably the endosomal targeting signal includes an endosomal targeting portion of human  
10 invariant chain Ii.

According to another aspect of the invention, an isolated nucleic acid encoding any of the foregoing HLA class II-binding peptides is provided. Preferably the nucleic acid comprises SEQ ID NO:43, SEQ ID NO:44 or SEQ ID NO:87.

According to still another aspect of the invention, expression vectors are provided.  
15 The expression vectors comprise any of the foregoing isolated nucleic acids operably linked to a promoter. In preferred embodiments, the nucleic acid comprises SEQ ID NO:43, SEQ ID NO:44 or SEQ ID NO:87. In other embodiments, the expression vector further comprise a nucleic acid which encodes an HLA-DRB1/13 molecule or an HLA-DPB1 molecule.

According to yet another aspect of the invention, host cells transfected or transformed  
20 with any of the foregoing expression vectors are provided. Host cells which express an HLA-DRB1/13 molecule or an HLA-DPB1 molecule, and which are transfected or transformed with any of the foregoing expression vectors are also provided.

According to another aspect of the invention, methods for enriching selectively a population of T lymphocytes with CD4<sup>+</sup> T lymphocytes specific for a MAGE-A3 HLA class  
25 II-binding peptide are provided. The methods include contacting an isolated population of T lymphocytes with an agent presenting a complex of the MAGE-A3 HLA class II-binding peptide and an HLA class II molecule in an amount sufficient to selectively enrich the isolated population of T lymphocytes with the CD4<sup>+</sup> T lymphocytes. In certain embodiments, the agent is an antigen presenting cell contacted with a MAGE-A3 protein or an HLA class II  
30 binding fragment thereof. In preferred embodiments, the HLA class II molecule is an HLA-DRB1/13 molecule or an HLA-DPB1 molecule and the MAGE-A3 HLA class II-binding peptide is a peptide having the amino acid sequence of any of SEQ ID NO:24, SEQ ID

NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 or SEQ ID NO:86. More preferably, the MAGE-A3 HLA class II-binding peptide includes the amino acid sequence of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 or SEQ ID NO:86. In certain embodiments of the foregoing methods, the isolated MAGE-A3 protein or HLA class II binding peptide thereof includes an endosomal targeting signal. Preferably the endosomal targeting signal includes an endosomal targeting portion of human invariant chain Ii.

According to a further aspect of the invention, methods for diagnosing a disorder characterized by expression of MAGE-A3 are provided. The methods include contacting a biological sample isolated from a subject with an agent that is specific for the MAGE-A3 HLA class II binding peptide, and determining the interaction between the agent and the MAGE-A3 HLA class II binding peptide as a determination of the disorder. The biological sample in some embodiments is, for example, dendritic cells loaded with a tumor cell lysate. In certain embodiments, the peptide comprises the amino acid sequence of SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:86, or a functional variant thereof. In preferred embodiments, the MAGE-A3 HLA class II-binding peptide is a peptide having the amino acid sequence of any of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86. More preferably, the MAGE-A3 HLA class II-binding peptide includes the amino acid sequence of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 or SEQ ID NO:86.

According to another aspect of the invention, methods for diagnosing a disorder characterized by expression of a MAGE-A3 HLA class II-binding peptide which forms a complex with an HLA class II molecule are provided. The methods include contacting a biological sample isolated from a subject with an agent that binds the complex; and determining binding between the complex and the agent as a determination of the disorder. In some embodiments the HLA class II molecule is an HLA-DRB1/13 molecule, such as HLA-DRB1/1301 or HLA-DRB1/1302, or an HLA-DPB1 molecule, such as HLA-DPB1\*0401, and the MAGE-A3 HLA class II-binding peptide comprises the amino

acid sequence of SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:86, or a functional variant thereof. Preferably the MAGE-A3 HLA class II-binding peptide is a peptide having the amino acid sequence of any of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86. More preferably, the MAGE-A3 HLA class II-binding peptide includes the amino acid sequence of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 or SEQ ID NO:86.

10           Methods for treating a subject having a disorder characterized by expression of MAGE-A3 are provided in another aspect of the invention. The methods include administering to the subject an amount of a MAGE-A3 HLA class II-binding peptide sufficient to ameliorate the disorder. In certain embodiments the MAGE-A3 HLA class II-binding peptide comprises the amino acid sequence of SEQ ID NO:41, SEQ ID NO:42 or  
15           SEQ ID NO:86, or a functional variant thereof. Preferably the MAGE-A3 HLA class II-binding peptide is a peptide having the amino acid sequence of any of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83,  
20           SEQ ID NO:84 and SEQ ID NO:86. More preferably, the MAGE-A3 HLA class II-binding peptide includes the amino acid sequence of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 or SEQ ID NO:86. In certain embodiments, the MAGE-A3 HLA class II binding peptide comprises an endosomal targeting signal, preferably an endosomal targeting portion of human invariant chain Ii.

25           According to still another aspect of the invention, methods for treating a subject having a disorder characterized by expression of MAGE-A3 are provided. The methods include administering to the subject an amount of a MAGE-A3 HLA class I-binding peptide and an amount of a MAGE-A3 HLA class II-binding peptide sufficient to ameliorate the disorder. In certain embodiments, the MAGE-A3 HLA class II-binding peptide comprises the  
30           amino acid sequence of SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:86, or a functional variant thereof. Preferably the MAGE-A3 HLA class II-binding peptide is a peptide having the amino acid sequence of any of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID

NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86. More preferably, the MAGE-A3 HLA class II-binding peptide includes the amino acid  
5 sequence of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 or SEQ ID NO:86. In certain embodiment of the foregoing methods, the MAGE-A3 HLA class I-binding peptide and the MAGE-A3 HLA class II-binding peptide are combined as a polytope polypeptide. In still other embodiments, the MAGE-A3 HLA class II binding peptide comprises an endosomal targeting signal, preferably an endosomal targeting portion of human  
10 invariant chain Ii.

According to yet another aspect of the invention, methods for treating a subject having a disorder characterized by expression of MAGE-A3 are provided. The methods include administering to the subject an amount of an agent which enriches selectively in the subject the presence of complexes of an HLA class II molecule and a MAGE-A3 HLA class II-  
15 binding peptide, sufficient to ameliorate the disorder. Preferably the HLA class II molecule is an HLA-DRB1/13 molecule, such as HLA-DRB1/1301 or HLA-DRB1/1302, or an HLA-DPB1 molecule, such as HLA-DPB1\*0401. In certain embodiments, the MAGE-A3 HLA class II-binding peptide comprises the amino acid sequence of SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:86, or a functional variant thereof. Preferably the MAGE-A3 HLA class  
20 II-binding peptide is a peptide having the amino acid sequence of any of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86. More preferably, the MAGE-A3 HLA class II-binding  
25 peptide includes the amino acid sequence of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 or SEQ ID NO:86. In certain embodiments, the agent comprises a MAGE-A3 HLA class II binding peptide. Preferably the MAGE-A3 HLA class II binding peptide includes an endosomal targeting signal. Preferred endosomal targeting signals include endosomal targeting portions of human invariant chain Ii.

30 Additional methods for treating a subject having a disorder characterized by expression of MAGE-A3 are provided in another aspect of the invention. The methods include administering to the subject an amount of autologous CD4<sup>+</sup> T lymphocytes sufficient

to ameliorate the disorder, wherein the CD4<sup>+</sup> T lymphocytes are specific for complexes of an HLA class II molecule and a MAGE-A3 HLA class II-binding peptide. Preferably the HLA class II molecule is an HLA-DRB1/13 molecule, such as HLA-DRB1/1301 or HLA-DRB1/1302, or an HLA-DPB1 molecule, such as HLA-DPB1\*0401. In certain  
5 embodiments, the MAGE-A3 HLA class II-binding peptide comprises the amino acid sequence of SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:86, or a functional variant thereof. Preferably the MAGE-A3 HLA class II-binding peptide is a peptide having the amino acid sequence of any of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32,  
10 SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86. More preferably, the MAGE-A3 HLA class II-binding peptide includes the amino acid sequence of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 or SEQ ID NO:86.

Methods for identifying functional variants of a MAGE-A3 HLA class II binding  
15 peptide are provided according to another aspect of the invention. According to the methods, a MAGE-A3 HLA class II binding peptide, an HLA class II binding molecule which binds the MAGE-A3 HLA class II binding peptide, and a T cell which is stimulated by the MAGE-A3 HLA class II binding peptide presented by the HLA class II binding molecule are selected. A first amino acid residue of the MAGE-A3 HLA class II binding peptide is mutated to prepare  
20 a variant peptide. The binding of the variant peptide to HLA class II binding molecule and stimulation of the T cell are then determined, wherein binding of the variant peptide to the HLA class II binding molecule and stimulation of the T cell by the variant peptide presented by the HLA class II binding molecule indicates that the variant peptide is a functional variant. In preferred embodiments, the MAGE-A3 HLA class II binding peptide comprises the amino  
25 acid sequence of SEQ ID NO:41, SEQ ID NO:42 or SEQ ID NO:86. More preferably, the MAGE-A3 HLA class II-binding peptide is a peptide having the amino acid sequence of any of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID  
30 NO:41, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86. More preferably, the MAGE-A3 HLA class II-binding peptide includes the amino acid sequence of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 or SEQ ID NO:86. In certain embodiments, the

methods further include the step of comparing the stimulation of the T cell by the MAGE-A3 HLA class II binding peptide and the stimulation of the T cell by the functional variant as a determination of the effectiveness of the stimulation of the T cell by the functional variant.

According to another aspect of the invention, an isolated polypeptide is provided. The  
5 isolated polypeptide binds selectively a MAGE-A3 HLA class II-binding peptide, provided that the isolated polypeptide is not an HLA class II molecule. In certain embodiments, the isolated polypeptide is an antibody and preferably is a monoclonal antibody. In other embodiments, the isolated polypeptide is an antibody fragment selected from the group consisting of a Fab fragment, a F(ab)<sub>2</sub> fragment or a fragment including a CDR3 region  
10 selective for a MAGE-A3 HLA class II-binding peptide.

According to still another aspect of the invention, an isolated CD4<sup>+</sup> T lymphocyte is provided. The isolated CD4<sup>+</sup> T lymphocyte selectively binds a complex of an HLA class II molecule and a MAGE-A3 HLA class II-binding peptide. Preferably the HLA class II molecule is an HLA-DRB1/13 molecule, such as HLA-DRB1/1301 or HLA-DRB1/1302, or  
15 an HLA-DPB1 molecule, such as HLA-DPB1\*0401. In some embodiments the MAGE-A3 HLA class II-binding peptide comprises the amino acid sequence of SEQ ID NO:41, SEQ ID NO:42 or SEQ ID NO:86, or a functional variant thereof. Preferably the MAGE-A3 HLA class II-binding peptide is a peptide having the amino acid sequence of any of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID  
20 NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86. More preferably, the MAGE-A3 HLA class II-binding peptide includes the amino acid sequence of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 or SEQ ID NO:86.

According to still another aspect of the invention, an isolated antigen presenting cell is  
25 provided. The isolated antigen presenting cell comprises a complex of an HLA class II molecule and a MAGE-A3 HLA class II-binding peptide. Preferably the HLA class II molecule is an HLA-DRB1/13 molecule, such as HLA-DRB1/1301 or HLA-DRB1/1302, or an HLA-DPB1 molecule, such as HLA-DPB1\*0401. In certain embodiments the MAGE-A3  
30 HLA class II-binding peptide comprises the amino acid sequence of SEQ ID NO:41, SEQ ID NO:42 or SEQ ID NO:86, or a functional variant thereof. Preferably the MAGE-A3 HLA class II-binding peptide is a peptide having the amino acid sequence of any of SEQ ID NO:24,

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SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86. More preferably, the MAGE-A3 HLA class II-binding peptide includes the amino acid sequence of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 or SEQ ID NO:86.

The invention also provides pharmaceutical preparations containing any one or more of the medicaments described above or throughout the specification. Such pharmaceutical preparations can include pharmaceutically acceptable diluent carriers or excipients.

The use of the foregoing compositions, peptides and nucleic acids in the preparation of a medicament, particularly a medicament for treatment of cancer, also is provided.

These and other objects of the invention will be described in further detail in connection with the detailed description of the invention.

#### **Brief Description of the Drawings**

Fig. 1 is a schematic representation of the protocol used to obtain CD4 T cell lines specific for MAGE-A3.

Fig. 2 is a graph showing CD4<sup>+</sup> T cell lines B6 and F3 recognized autologous EBV-B cells which have processed the recombinant His-MAGE-A3 protein.

Fig. 3 is a graph showing that the recognition by CD4<sup>+</sup> T cell clones of autologous EBV-B cells pulsed with exogenous His-MAGE-A3 protein is inhibited by an anti-HLA DR monoclonal antibody.

Fig. 4 is a graph detailing the screening of MAGE-A3 peptides for recognition by CD4<sup>+</sup> clones 436/B6.34 (B6/34), 436/B6.37 (B6/37), F3/37 and F3/40.

Fig. 5 is a graph depicting stimulation of TNF and IFN- $\gamma$  production by CD4<sup>+</sup> clones 436/B6.34 (B6/34) and 436/B6.37 (B6/37) EBV-B cells pulsed with the peptide RKVAELVHFLLLKYRA (MAGE-A3<sub>111-126</sub>, SEQ ID NO:3) or ELVHFLLLKYRAREPV (MAGE-A3<sub>115-130</sub>, SEQ ID NO:4).

Fig. 6 is a graph which shows that autologous EBV-B cells pulsed with the peptide MAGE-A3<sub>111-126</sub> or MAGE-A3<sub>115-130</sub> induced the proliferation of clones 436/B6.34 (B6/34), 436/B6.37 (B6/37).

Fig. 7 is a graph which demonstrates that the response of CD4<sup>+</sup> clone 436/B6.37

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(B6/37) to peptide MAGE-A3<sub>115-130</sub> is HLA-DRB1/1302 restricted.

Fig. 8 is a graph which shows the reactivity of clone 436/B6.37 (B6/37) against autologous EBV-B cells pulsed with truncated peptides derived from MAGE-A3<sub>115-130</sub>.

Fig. 9 depicts the presentation of exogenous MAGE-A3 processed by dendritic cells to  
5 clone 37.

Fig. 10 shows the recognition of transduced MZ2 EBV by CD4 T cell clone 436/B6.37 (anti-MAGE-A3.DR13).

Fig. 11 shows the recognition of transduced MZ2 EBV by CTL clone 434/1 (anti-MAGE-A3.A1).

10 Fig. 12 shows the lysis of transduced MZ2 EBV by CTL434/1 (anti-MAGE-A3.A1).

Fig. 13 shows the recognition of transduced MZ2-MEL.43 by CD4 T cell clone 436/B6.37 (anti-MAGE-A3.DR13) and CTL clone 434/1 (anti-MAGE-A3.A1).

Fig. 14 is a schematic drawing of retroviral constructs of invariant chain (Ii) and LAMP-1 MAGE-A3 fusion proteins.

15 Fig. 15 shows that clones 2 and 22 recognized a MAGE-A3 derived peptide in association with HLA-DR.

Fig. 16 shows an overview of the procedure used to obtain anti-MAGE-A3 CD4<sup>+</sup> cell clones using MAGE-A3 (insect) and MAGE-A3 (bacteria) proteins.

Fig. 17 depicts production of IFN- $\gamma$  by CD4<sup>+</sup> T cells stimulated with autologous  
20 EBV-B cells incubated with protein MAGE-A3.

Fig. 18 demonstrates that MAGE-A3 peptides recognized by CD4 clones 43 and 22. (A) Stimulation of clone C3.43 by two overlapping MAGE-A3 peptides. (B) Titration of the shortest peptide recognized by clone C3.22 (TQHFVQENYLEY; SEQ ID NO:86).

Fig. 19 shows that the MAGE-A3 peptide is presented to clone C3.22 by HLA-DP  
25 molecules.

Fig. 20 demonstrates that HLA-DPB1\*0401 tumor cells expressing MAGE-A3 are recognized by CD4 clones C3.22 and C3.43.

Fig. 21 shows the lytic activity of CD4 clone C3.22.

### 30 Detailed Description of the Invention

The invention provides isolated MAGE-A3 peptides presented by HLA class II molecules, which peptides stimulate the proliferation and activation of CD4<sup>+</sup> T lymphocytes.

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Such peptides are referred to herein as "MAGE-A3 HLA class II binding peptides", "HLA class II binding peptides" and "MHC class II binding peptides". Hence, one aspect of the invention is an isolated peptide which includes the amino acid sequence of SEQ ID NO:41, SEQ ID NO:42 or SEQ ID NO:86.

5           The examples below show the isolation of peptides which are MAGE-A3 HLA class II binding peptides. These exemplary peptides are processed translation products of the nucleic acid of SEQ ID NO:1. As such, it will be appreciated by one of ordinary skill in the art that the translation products from which a MAGE-A3 HLA class II binding peptide is processed to a final form for presentation may be of any length or sequence so long as they encompass the  
10 MAGE-A3 HLA class II binding peptide. As demonstrated in the examples below, peptides or proteins as small as 10 amino acids and as large as the amino acid sequence of the MAGE-A3 protein (SEQ ID NO:2) are appropriately processed, presented by HLA class II molecules and effective in stimulating CD4<sup>+</sup> T lymphocytes. MAGE-A3 HLA class II binding peptides, such as the peptides of SEQ ID NO:41, SEQ ID NO:42 or SEQ ID NO:86 may have one, two,  
15 three, four, five, six, seven, eight, nine, ten, or more amino acids added to either or both ends. The antigenic portion of such a peptide is cleaved out under physiological conditions for presentation by HLA class II molecules. It is also well known in the art that HLA class II peptide length is variable between about 10 amino acids and about 30 amino acids (Engelhard, *Ann. Rev. Immunol.* 12:181-201, 1994). Most of the HLA class II binding peptides fall in to  
20 the length range of 12-19 amino acids. Nested sets of HLA class II binding peptides have been identified, wherein the peptides share a core sequence but have different amino acids at amino and/or carboxyl terminal ends (*see, e.g., Chicz et al., J. Exp. Med.* 178:27-47, 1993). Thus additional MAGE-A3 HLA class II binding peptides, as well as MAGE family HLA class II binding peptides, can be identified by one of ordinary skill in the art according to the  
25 procedures described herein.

          The procedures described in the Examples can be utilized to identify MAGE family HLA class II binding peptides. Thus, for example, one can load antigen presenting cells, such as dendritic cells of normal blood donors, with a recombinant MAGE protein (or a fragment thereof) by contacting the cells with the MAGE polypeptide or by introducing into the cells a  
30 nucleic acid molecule which directs the expression of the MAGE protein of interest. The antigen-presenting cells then can be used to induce *in vitro* the activation and proliferation of specific CD4 lymphocytes which recognize MAGE HLA class II binding peptides. The

sequence of the peptides then can be determined as described in the Examples, e.g., by stimulating cells with peptide fragments of the MAGE protein used to stimulate the activation and proliferation of CD4 lymphocytes. Alternatively, one can load antigen presenting cells with peptides derived from a MAGE protein. For example, one can make predictions of peptide sequences derived from MAGE family proteins which are candidate HLA class II binding peptides based on the consensus amino acid sequences for binding HLA class II molecules. In this regard, see, e.g. International applications PCT/US96/03182 and PCT/US98/01373. Peptides which are thus selected can be used in the assays described herein for inducing specific CD4 lymphocytes and identification of peptides. Additional methods of selecting and testing peptides for HLA class II binding are well known in the art.

As noted above, the invention embraces functional variants of MAGE-A3 HLA class II binding peptides. As used herein, a "functional variant" or "variant" of a HLA class II binding peptide is a peptide which contains one or more modifications to the primary amino acid sequence of a HLA class II binding peptide and retains the HLA class II and T cell receptor binding properties disclosed herein. Modifications which create a MAGE-A3 HLA class II binding peptide functional variant can be made for example 1) to enhance a property of a MAGE-A3 HLA class II binding peptide, such as peptide stability in an expression system or the stability of protein-protein binding such as HLA-peptide binding; 2) to provide a novel activity or property to a MAGE-A3 HLA class II binding peptide, such as addition of an antigenic epitope or addition of a detectable moiety; or 3) to provide a different amino acid sequence that produces the same or similar T cell stimulatory properties. Modifications to MAGE-A3 (as well as MAGE family) HLA class II binding peptides can be made to nucleic acids which encodes the peptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety, such as biotin, addition of a fatty acid, substitution of one amino acid for another and the like. Variants also can be selected from libraries of peptides, which can be random peptides or peptides based on the sequence of the MAGE peptides including substitutions at one or more positions. For example, a peptide library can be used in competition assays with complexes of MAGE peptides bound to HLA class II molecules (e.g. dendritic cells loaded with MAGE peptide). Peptides which compete for binding of the MAGE peptide to the HLA class II molecule can be sequenced and used in other assays (e.g.

CD4 lymphocyte proliferation) to determine suitability as MAGE peptide functional variants.

Modifications also embrace fusion proteins comprising all or part of a MAGE HLA class II binding peptide amino acid sequence, such as the invariant chain-MAGE-A3 fusion proteins described herein. The invention thus embraces fusion proteins comprising MAGE-A3 HLA class II binding peptides and endosomal targeting signals such as the human invariant chain (Ii). As is disclosed below, fusion of an endosomal targeting portion of the human invariant chain to MAGE-A3 resulted in efficient targeting of MAGE-A3 to the HLA class II peptide presentation pathway. An "endosomal targeting portion" of the human invariant chain or other targeting polypeptide is that portion of the molecule which, when fused or conjugated to a second polypeptide, increases endosomal localization of the second polypeptide. Thus endosomal targeting portions can include the entire sequence or only a small portion of a targeting polypeptide such as human invariant chain Ii. One of ordinary skill in the art can readily determine an endosomal targeting portion of a targeting molecule.

Surprisingly, fusion of an endosomal targeting portion of LAMP-1 protein did not significantly increase targeting of MAGE-A3 to the HLA class II peptide presentation pathway. Therefore, the invention includes the unexpected finding that fusion proteins of MAGE-A3 and human invariant chain Ii, but not LAMP-1, are efficiently targeted to the HLA class II peptide presentation pathway. Additional endosomal targeting signals can be identified by one of ordinary skill in the art, fused to MAGE-A3 or a MAGE-A3 HLA class II binding portion thereof, and tested for targeting to the HLA class II peptide presentation pathway using no more than routine experimentation.

The amino acid sequence of MAGE HLA class II binding peptides may be of natural or non-natural origin, that is, they may comprise a natural MAGE HLA class II binding peptide molecule or may comprise a modified sequence as long as the amino acid sequence retains the ability to stimulate helper T cells when presented and retains the property of binding to an HLA class II molecule such as an HLA DRB1/13 molecule or an HLA DPB1 molecule. For example, MAGE-A3 HLA class II binding peptides in this context may be fusion proteins including a MAGE-A3 HLA class II binding peptide and unrelated amino acid sequences, synthetic peptides of amino acid sequences shown in SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83,

SEQ ID NO:84 and SEQ ID NO:86, labeled peptides, peptides isolated from patients with a MAGE-A3 expressing cancer, peptides isolated from cultured cells which express MAGE-A3, peptides coupled to nonpeptide molecules (for example in certain drug delivery systems) and other molecules which include the amino acid sequence of SEQ ID NO:41, SEQ ID NO:42 or  
5 SEQ ID NO:86.

Preferably, MAGE HLA class II binding peptides are non-hydrolyzable. To provide such peptides, one may select MAGE HLA class II binding peptides from a library of non-hydrolyzable peptides, such as peptides containing one or more D-amino acids or peptides containing one or more non-hydrolyzable peptide bonds linking amino acids. Alternatively,  
10 one can select peptides which are optimal for inducing CD4<sup>+</sup> T lymphocytes and then modify such peptides as necessary to reduce the potential for hydrolysis by proteases. For example, to determine the susceptibility to proteolytic cleavage, peptides may be labeled and incubated with cell extracts or purified proteases and then isolated to determine which peptide bonds are susceptible to proteolysis, e.g., by sequencing peptides and proteolytic fragments.

15 Alternatively, potentially susceptible peptide bonds can be identified by comparing the amino acid sequence of a MAGE-A3 HLA class II binding peptide with the known cleavage site specificity of a panel of proteases. Based on the results of such assays, individual peptide bonds which are susceptible to proteolysis can be replaced with non-hydrolyzable peptide bonds by *in vitro* synthesis of the peptide.

20 Many non-hydrolyzable peptide bonds are known in the art, along with procedures for synthesis of peptides containing such bonds. Non-hydrolyzable bonds include -psi[CH<sub>2</sub>NH]- reduced amide peptide bonds, -psi[COCH<sub>2</sub>]- ketomethylene peptide bonds, -psi[CH(CN)NH]- (cyanomethylene)amino peptide bonds, -psi[CH<sub>2</sub>CH(OH)]- hydroxyethylene peptide bonds, -psi[CH<sub>2</sub>O]- peptide bonds, and -psi[CH<sub>2</sub>S]- thiomethylene peptide bonds.

25 Nonpeptide analogs of peptides, e.g., those which provide a stabilized structure or lessened biodegradation, are also contemplated. Peptide mimetic analogs can be prepared based on a selected MAGE-A3 HLA class II binding peptide by replacement of one or more residues by nonpeptide moieties. Preferably, the nonpeptide moieties permit the peptide to retain its natural conformation, or stabilize a preferred, e.g., bioactive, conformation. Such  
30 peptides can be tested in molecular or cell-based binding assays to assess the effect of the substitution(s) on conformation and/or activity. One example of methods for preparation of nonpeptide mimetic analogs from peptides is described in Nachman et al., *Regul. Pept.*

57:359-370 (1995). Peptide as used herein embraces all of the foregoing.

If a variant involves a change to an amino acid of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, 5 SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 or SEQ ID NO:86, functional variants of the MAGE-A3 HLA class II binding peptide having conservative amino acid substitutions typically will be preferred, i.e., substitutions which retain a property of the original amino acid such as charge, hydrophobicity, conformation, etc. Examples of conservative substitutions of amino acids include 10 substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Other methods for identifying functional variants of the MAGE-A3 HLA class II binding peptides are provided in a published PCT application of Strominger and Wucherpennig (PCT/US96/03182). These methods rely upon the development of amino acid 15 sequence motifs to which potential epitopes may be compared. Each motif describes a finite set of amino acid sequences in which the residues at each (relative) position may be (a) restricted to a single residue, (b) allowed to vary amongst a restricted set of residues, or (c) allowed to vary amongst all possible residues. For example, a motif might specify that the residue at a first position may be any one of the residues valine, leucine, isoleucine, 20 methionine, or phenylalanine; that the residue at the second position must be histidine; that the residue at the third position may be any amino acid residue; that the residue at the fourth position may be any one of the residues valine, leucine, isoleucine, methionine, phenylalanine, tyrosine or tryptophan; and that the residue at the fifth position must be lysine.

Sequence motifs for MAGE-A3 HLA class II binding peptide functional variants can 25 be developed by analysis of the binding domains or binding pockets of major histocompatibility complex HLA-DR proteins and/or the T cell receptor ("TCR") contact points of the MAGE-A3 HLA class II binding peptides disclosed herein. By providing a detailed structural analysis of the residues involved in forming the HLA class II binding pockets, one is enabled to make predictions of sequence motifs for binding of MAGE peptides 30 to any of the HLA class II proteins.

Using these sequence motifs as search, evaluation, or design criteria, one is enabled to identify classes of peptides (e.g. MAGE HLA class II binding peptides, particularly the

MAGE-A3 peptides disclosed herein, and functional variants thereof) which have a reasonable likelihood of binding to a particular HLA molecule and of interacting with a T cell receptor to induce T cell response. These peptides can be synthesized and tested for activity as described herein. Use of these motifs, as opposed to pure sequence homology (which  
5 excludes many peptides which are antigenically similar but quite distinct in sequence) or sequence homology with unlimited "conservative" substitutions (which admits many peptides which differ at critical highly conserved sites), represents a method by which one of ordinary skill in the art can evaluate peptides for potential application in the treatment of disease.

The Strominger and Wucherpennig PCT application, and references cited therein, all  
10 of which are incorporated by reference, describe the HLA class II and TCR binding pockets which contact residues of an HLA class II peptide. By keeping the residues which are likely to bind in the HLA class II and/or TCR binding pockets constant or permitting only specified substitutions, functional variants of MAGE HLA class II binding peptides can be prepared which retain binding to HLA class II and T cell receptor.

15 Thus methods for identifying additional MAGE family HLA class II peptides, in particular MAGE-A3 HLA class II binding peptides, and functional variants thereof, are provided. In general, any MAGE protein can be subjected to the analysis noted above, peptide sequences selected and the tested as described herein. With respect to MAGE-A3, for example, the methods include selecting a MAGE-A3 HLA class II binding peptide, an HLA  
20 class II binding molecule which binds the MAGE-A3 HLA class II binding peptide, and a T cell which is stimulated by the MAGE-A3 HLA class II binding peptide presented by the HLA class II binding molecule. In preferred embodiments, the MAGE-A3 HLA class II binding peptide comprises the amino acid sequence of SEQ ID NO:41, SEQ ID NO:42 or SEQ ID NO:86. More preferably, the peptide consists of the amino acid sequence of SEQ ID  
25 NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 or SEQ ID NO:86. A first amino acid residue of the MAGE-A3 HLA class II binding peptide is mutated to prepare a variant peptide. The amino acid  
30 residue can be mutated according to the principles of HLA and T cell receptor contact points set forth in the Strominger and Wucherpennig PCT application described above. Any method for preparing variant peptides can be employed, such as synthesis of the variant

peptide, recombinantly producing the variant peptide using a mutated nucleic acid molecule, and the like.

The binding of the variant peptide to HLA class II binding molecule and stimulation of the T cell are then determined according to standard procedures. For example, as exemplified  
5 below, the variant peptide can be contacted with an antigen presenting cell which contains the HLA class II molecule which binds the MAGE-A3 peptide to form a complex of the variant peptide and antigen presenting cell. This complex can then be contacted with a T cell which recognizes the MAGE-A3 HLA class II binding peptide presented by the HLA class II binding molecule. T cells can be obtained from a patient having a condition characterized by  
10 expression of MAGE-A3. Recognition of variant peptides by the T cells can be determined by measuring an indicator of T cell stimulation such as TNF or IFN $\gamma$  production. Similar procedures can be carried out for identification and characterization of other MAGE family HLA class II binding peptides.

Binding of a variant peptide to the HLA class II binding molecule and stimulation of  
15 the T cell by the variant peptide presented by the HLA class II binding molecule indicates that the variant peptide is a functional variant. The methods also can include the step of comparing the stimulation of the T cell by the MAGE-A3 HLA class II binding peptide and the stimulation of the T cell by the functional variant as a determination of the effectiveness of the stimulation of the T cell by the functional variant. By comparing the functional variant  
20 with the MAGE-A3 HLA class II binding peptide, peptides with increased T cell stimulatory properties can be prepared.

Variants of the MAGE-A3 HLA class II binding peptides prepared by any of the foregoing methods can be sequenced, if necessary, to determine the amino acid sequence and thus deduce the nucleotide sequence which encodes such variants.

25 Also a part of the invention are those nucleic acid sequences which code for a MAGE HLA class II binding peptides or variant thereof and other nucleic acid sequences which hybridize to a nucleic acid molecule consisting of the above described nucleotide sequences, under stringent conditions. The term "stringent conditions" as used herein refers to parameters with which the art is familiar. Nucleic acid hybridization parameters may be found in  
30 references which compile such methods, e.g. *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al.,

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eds., John Wiley & Sons, Inc., New York. More specifically, stringent conditions, as used herein, refers to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% Polyvinyl pyrrolidone, 0.02% Bovine Serum Albumin, 25mM NaH<sub>2</sub>PO<sub>4</sub> (pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M Sodium Chloride/0.15M Sodium Citrate, pH 7; SDS is Sodium Dodecyl Sulphate; and EDTA is Ethylene diaminetetraacetic acid. After hybridization, the membrane upon which the DNA is transferred is washed at 2xSSC at room temperature and then at 0.1 - 0.5 X SSC/0.1 X SDS at temperatures up to 65°C.

There are other conditions, reagents, and so forth which can be used, which result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of homologs and alleles of nucleic acids encoding the MAGE HLA class II binding peptides of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

In general homologs and alleles typically will share at least 50% amino acid identity and/or at least 40% nucleotide identity to the amino acid sequence of a MAGE-A3 HLA class II binding peptide (such as SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86) or nucleic acids which encode such a peptide, respectively. In some instances homologs and alleles will share at least 90% nucleotide identity and/or at least 95% amino acid identity and in still other instances will share at least 95% nucleotide identity and/or at least 99% amino acid identity. Complements of the foregoing nucleic acids also are embraced by the invention.

In screening for nucleic acids which encode a MAGE HLA class II binding peptide, a nucleic acid hybridization such as a Southern blot or a Northern blot may be performed using the foregoing conditions, together with a <sup>32</sup>P probe. After washing the membrane to which DNA encoding a MAGE HLA class II binding peptide was finally transferred, the membrane can be placed against X-ray film to detect the radioactive signal.

The invention also includes the use of nucleic acid sequences which include alternative

codons that encode the same amino acid residues of the MAGE HLA class II binding peptides. For example, as disclosed herein, the peptide RKVAELVHFLLKYRA (SEQ ID NO:3) is a MAGE-A3 HLA class II binding peptide. The leucine residues (amino acids No. 6, 10, 11 and 12 of SEQ ID NO:3) can be encoded by the codons CUA, CUC, CUG, CUU, UUA and UUG. Each of the six codons is equivalent for the purposes of encoding a leucine residue. Thus, it will be apparent to one of ordinary skill in the art that any of the leucine-encoding nucleotide triplets may be employed to direct the protein synthesis apparatus, *in vitro* or *in vivo*, to incorporate a leucine residue. Similarly, nucleotide sequence triplets which encode other amino acid residues comprising the MAGE-A3 HLA class II binding peptide of SEQ ID NO:3 include: CGA, CGC, CGG, CGT, AGA and AGG (arginine codons); AAA and AAG (lysine codons); GUA, GUC, GUG and GUU (valine codons); GAA and GAG (glutamine codons); CAC and CAU (histidine codons); UUC and UUU (phenylalanine codons) and UAC and UAU (tyrosine codons). Other amino acid residues may be encoded similarly by multiple nucleotide sequences. Thus, the invention embraces degenerate nucleic acids that differ from the native MAGE HLA class II binding peptide encoding nucleic acids in codon sequence due to the degeneracy of the genetic code.

It will also be understood that the invention embraces the use of the sequences in expression vectors, as well as to transfect host cells and cell lines, be these prokaryotic (e.g., *E. coli*), or eukaryotic (e.g., dendritic cells, CHO cells, COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). The expression vectors require that the pertinent sequence, i.e., those described *supra*, be operably linked to a promoter. As it has been found that human HLA-DRB1/1302 molecules present a MAGE-A3 HLA class II binding peptide, the expression vector may also include a nucleic acid sequence coding for an HLA-DRB1/13 molecule. (For other MAGE HLA class II binding peptides, different HLA molecules, such as HLA-DPB1 molecules, especially HLA-DPB1\*0401, can be used.) In a situation where the vector contains both coding sequences, it can be used to transfect a cell which does not normally express either one. The MAGE-A3 HLA class II binding peptide coding sequence may be used alone, when, e.g. the host cell already expresses an HLA-DRB1/13 molecule or an HLA-DPB1 molecule. Of course, there is no limit on the particular host cell which can be used as the vectors which contain the two coding sequences may be used in host cells which do not express HLA-DRB1/13 molecules or HLA-DPB1 molecules if desired, and the nucleic acid coding for the MAGE-A3 HLA class II binding peptide can be

used in antigen presenting cells which express an HLA-DRB1/13 molecule or an HLA-DPB1 molecule.

As used herein, "an HLA-DRB1/13 molecule" includes the subtypes DRB1\*1301, DRB1\*1302, DRB1\*13031, DRB1\*13032, DRB1\*1304, DRB1\*1305, DRB1\*1306,  
5 DRB1\*1307, DRB1\*1308, DRB1\*1309, DRB1\*1310, DRB1\*1311, DRB1\*1312, DRB1\*1314, DRB1\*1315, DRB1\*1316, DRB1\*1317, DRB1\*1318, DRB1\*1319, DRB1\*1320, DRB1\*1321, DRB1\*1322, DRB1\*1323 and DRB1\*1324. An HLA-DRB1/13 molecule also includes the subtypes which can be found in Bodmer et al., *Tissue Antigens* 49:297, 1996.

10 As used herein, "an HLA-DPB1 molecule" includes the subtypes DPB1\*01012, DPB1\*02011, DPB1\*02012, DPB1\*02013, DPB1\*0202, DPB1\*0301, DPB1\*0401, DPB1\*0402, DPB1\*0501, DPB1\*0601, DPB1\*0701, DPB1\*0801, DPB1\*0901, DPB1\*1001, DPB1\*11011, DPB1\*11012, DPB1\*1201, DPB1\*1301, DPB1\*1401, DPB1\*1501, DPB1\*1601, DPB1\*1701, DPB1\*1801, DPB1\*1901, DPB1\*20011,  
15 DPB1\*20012, DPB1\*2101, DPB1\*2201, DPB1\*2301, DPB1\*2401, DPB1\*2501, DPB1\*26011, DPB1\*26012, DPB1\*2701, DPB1\*2801, DPB1\*2901, DPB1\*3001, DPB1\*3101, DPB1\*3201, DPB1\*3301, DPB1\*3401, DPB1\*3501, DPB1\*3601, DPB1\*3701, DPB1\*3801, DPB1\*3901, DPB1\*4001, DPB1\*4101, DPB1\*4201, DPB1\*4301, DPB1\*4401, DPB1\*4501, DPB1\*4601, DPB1\*4701, DPB1\*4801,  
20 DPB1\*4901, DPB1\*5001, DPB1\*5101, DPB1\*5201, DPB1\*5301, DPB1\*5401, DPB1\*5501, DPB1\*5601, DPB1\*5701, DPB1\*5801, DPB1\*5901, DPB1\*6001, DPB1\*6101N, DPB1\*6201, DPB1\*6301, DPB1\*6401N, DPB1\*6501, DPB1\*6601, DPB1\*6701, DPB1\*6801, DPB1\*6901, DPB1\*7001, DPB1\*7101, DPB1\*7201, DPB1\*7301, DPB1\*7401, DPB1\*7501, DPB1\*7601, DPB1\*7701, DPB1\*7801,  
25 DPB1\*7901, DPB1\*8001, DPB1\*8101, DPB1\*8401 and other subtypes known to one of ordinary skill in the art. Preferably, an HLA-DPB1 molecule is an HLA-DPB1\*04 molecule, particularly HLA-DPB1\*0401.

As used herein, a "vector" may be any of a number of nucleic acids into which a desired sequence may be inserted by restriction and ligation for transport between different  
30 genetic environments or for expression in a host cell. Vectors are typically composed of DNA although RNA vectors are also available. Vectors include, but are not limited to, plasmids, phagemids and virus genomes. A cloning vector is one which is able to replicate

autonomously or after integration into the genome in a host cell, and which is further characterized by one or more endonuclease restriction sites at which the vector may be cut in a determinable fashion and into which a desired DNA sequence may be ligated such that the new recombinant vector retains its ability to replicate in the host cell. In the case of plasmids, replication of the desired sequence may occur many times as the plasmid increases in copy number within the host bacterium or just a single time per host before the host reproduces by mitosis. In the case of phage, replication may occur actively during a lytic phase or passively during a lysogenic phase. An expression vector is one into which a desired DNA sequence may be inserted by restriction and ligation such that it is operably joined to regulatory sequences and may be expressed as an RNA transcript. Vectors may further contain one or more marker sequences suitable for use in the identification of cells which have or have not been transformed or transfected with the vector. Markers include, for example, genes encoding proteins which increase or decrease either resistance or sensitivity to antibiotics or other compounds, genes which encode enzymes whose activities are detectable by standard assays known in the art (e.g.,  $\beta$ -galactosidase, luciferase or alkaline phosphatase), and genes which visibly affect the phenotype of transformed or transfected cells, hosts, colonies or plaques (e.g., green fluorescent protein). Preferred vectors are those capable of autonomous replication and expression of the structural gene products present in the DNA segments to which they are operably joined.

Preferably the expression vectors contain sequences which target a MAGE family polypeptide, e.g. MAGE-A3, or a HLA class II binding peptide derived therefrom, to the endosomes of a cell in which the protein or peptide is expressed. HLA class II molecules contain an invariant chain (Ii) which impedes binding of other molecules to the HLA class II molecules. This invariant chain is cleaved in endosomes, thereby permitting binding of peptides by HLA class II molecules. Therefore it is preferable that the MAGE-A3 HLA class II binding peptides and precursors thereof (e.g. the MAGE-A3 protein) are targeted to the endosome, thereby enhancing MAGE-A3 HLA class II binding peptide binding to HLA class II molecules. Targeting signals for directing molecules to endosomes are known in the art and these signals conveniently can be incorporated in expression vectors such that fusion proteins which contain the endosomal targeting signal are produced. Sanderson et al. (*Proc. Nat'l. Acad. Sci. USA* 92:7217-7221, 1995), Wu et al. (*Proc. Nat'l. Acad. Sci. USA* 92:11671-11675, 1995) and Thomson et al. (*J. Virol.* 72:2246-2252, 1998) describe endosomal targeting signals

(including invariant chain Ii and lysosomal-associated membrane protein LAMP-1) and their use in directing antigens to endosomal and/or lysosomal cellular compartments. As disclosed in the Examples, invariant chain-MAGE-A3 fusion proteins are preferred.

Endosomal targeting signals such as invariant chain also can be conjugated to MAGE-A3 protein or peptides by non-peptide bonds (i.e. not fusion proteins) to prepare a conjugate capable of specifically targeting MAGE-A3. Specific examples of covalent bonds include those wherein bifunctional cross-linker molecules are used. The cross-linker molecules may be homobifunctional or heterobifunctional, depending upon the nature of the molecules to be conjugated. Homobifunctional cross-linkers have two identical reactive groups.

Heterobifunctional cross-linkers are defined as having two different reactive groups that allow for sequential conjugation reaction. Various types of commercially available cross-linkers are reactive with one or more of the following groups; primary amines, secondary amines, sulfhydryls, carboxyls, carbonyls and carbohydrates. One of ordinary skill in the art will be able to ascertain without undue experimentation the preferred molecule for linking the endosomal targeting moiety and MAGE-A3 peptide or protein, based on the chemical properties of the molecules being linked and the preferred characteristics of the bond or bonds.

As used herein, a coding sequence and regulatory sequences are said to be "operably" joined when they are covalently linked in such a way as to place the expression or transcription of the coding sequence under the influence or control of the regulatory sequences. If it is desired that the coding sequences be translated into a functional protein, two DNA sequences are said to be operably joined if induction of a promoter in the 5' regulatory sequences results in the transcription of the coding sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the coding sequences, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a promoter region would be operably joined to a coding sequence if the promoter region were capable of effecting transcription of that DNA sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

The precise nature of the regulatory sequences needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribed and 5' non-translated sequences involved with the initiation of transcription and translation

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respectively, such as a TATA box, capping sequence, CAAT sequence, and the like.

Especially, such 5' non-transcribed regulatory sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined gene.

Regulatory sequences may also include enhancer sequences or upstream activator sequences

5 as desired. The vectors of the invention may optionally include 5' leader or signal sequences. The choice and design of an appropriate vector is within the ability and discretion of one of ordinary skill in the art.

Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., Sambrook et al.,  
10 *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding a MAGE-A3 HLA class II binding peptide. That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous DNA in the host cell. As described herein, such  
15 expression constructs optionally also contain nucleotide sequences which encode endosomal targeting signals, preferably human invariant chain or a targetting fragment thereof

Preferred systems for mRNA expression in mammalian cells are those such as pRc/CMV (available from Invitrogen, Carlsbad, CA) that contain a selectable marker such as a gene that confers G418 resistance (which facilitates the selection of stably transfected cell  
20 lines) and the human cytomegalovirus (CMV) enhancer-promoter sequences. Additionally, suitable for expression in primate or canine cell lines is the pCEP4 vector (Invitrogen), which contains an Epstein Barr virus (EBV) origin of replication, facilitating the maintenance of plasmid as a multicopy extrachromosomal element. Another expression vector is the pEF-BOS plasmid containing the promoter of polypeptide Elongation Factor 1 $\alpha$ , which stimulates  
25 efficiently transcription *in vitro*. The plasmid is described by Mishizuma and Nagata (*Nuc. Acids Res.* 18:5322, 1990), and its use in transfection experiments is disclosed by, for example, Demoulin (*Mol. Cell. Biol.* 16:4710-4716, 1996). Still another preferred expression vector is an adenovirus, described by Stratford-Perricaudet, which is defective for E1 and E3 proteins (*J. Clin. Invest.* 90:626-630, 1992). The use of the adenovirus as an Adeno.P1A  
30 recombinant is disclosed by Warnier et al., in intradermal injection in mice for immunization against P1A (*Int. J. Cancer*, 67:303-310, 1996).

The invention also embraces so-called expression kits, which allow the artisan to

prepare a desired expression vector or vectors. Such expression kits include at least separate portions of at least two of the previously discussed materials. Other components may be added, as desired.

The invention as described herein has a number of uses, some of which are described herein. The following uses are described for MAGE-A3 HLA class II binding peptides but are equally applicable to use of other MAGE family HLA class II binding peptides. First, the invention permits the artisan to diagnose a disorder characterized by expression of a MAGE-A3 HLA class II binding peptide. These methods involve determining expression of a MAGE-A3 HLA class II binding peptide, or a complex of a MAGE-A3 HLA class II binding peptide and an HLA class II molecule in a biological sample. The expression of a peptide or complex of peptide and HLA class II molecule can be determined by assaying with a binding partner for the peptide or complex, such as an antibody.

The invention also permits the artisan to treat a subject having a disorder characterized by expression of a MAGE-A3 HLA class II binding peptide. Treatments include administering an agent which enriches in the subject a complex of a MAGE-A3 HLA class II binding peptide and an HLA class II molecule, and administering CD4<sup>+</sup> T lymphocytes which are specific for such complexes. Agents useful in the foregoing treatments include MAGE-A3 HLA class II binding peptides and functional variants thereof, endosome-targeted fusion proteins which include such MAGE-A3 peptides, nucleic acids which express such proteins and peptides (including viruses which contain the nucleic acids), complexes of such peptides and HLA class II binding molecules (e.g. HLA DRB1/1302, HLA-DPB1\*0401), antigen presenting cells bearing complexes of a MAGE-A3 HLA class II binding peptide and an HLA class II binding molecule, and the like. The invention also permits an artisan to selectively enrich a population of T lymphocytes for CD4<sup>+</sup> T lymphocytes specific for a MAGE-A3 HLA class II binding peptide.

The isolation of the MAGE-A3 HLA class II binding peptides also makes it possible to isolate nucleic acids which encode the MAGE-A3 HLA class II binding peptides. Nucleic acids can be used to produce *in vitro* or in prokaryotic or eukaryotic host cells the MAGE-A3 HLA class II binding peptides. A variety of methodologies well-known to the skilled practitioner can be utilized to obtain isolated MAGE-A3 HLA class II binding peptides. For example, an expression vector may be introduced into cells to cause production of the peptides. In another method, mRNA transcripts may be microinjected or otherwise

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introduced into cells to cause production of the encoded peptides. Translation of mRNA in cell-free extracts such as the reticulocyte lysate system also may be used to produce peptides. Peptides comprising the MAGE-A3 HLA class II binding peptide of the invention may also be synthesized *in vitro*. Those skilled in the art also can readily follow known methods for isolating peptides in order to obtain isolated MAGE-A3 HLA class II binding peptides. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange chromatography and immune-affinity chromatography.

These isolated MAGE-A3 HLA class II binding peptides, proteins which include such peptides, or complexes of the peptides and HLA class II molecules, such as an HLA-DRB1/13 molecule or an HLA-DPB1 molecule, may be combined with materials such as adjuvants to produce vaccines useful in treating disorders characterized by expression of the MAGE-A3 HLA class II binding peptide. In addition, vaccines can be prepared from cells which present the MAGE-A3 HLA class II binding peptide/HLA complexes on their surface, such as dendritic cells, B cells, non-proliferative transfectants, etcetera. In all cases where cells are used as a vaccine, these can be cells transfected with coding sequences for one or both of the components necessary to stimulate CD4<sup>+</sup> lymphocytes, or be cells which already express both molecules without the need for transfection. For example, autologous antigen presenting cells can be isolated from a patient and treated to obtain cells which present MAGE-A3 epitopes in association of HLA class I and HLA class II molecules. These cells would be capable of stimulating both CD4<sup>+</sup> and CD8<sup>+</sup> cell responses. Such antigen presenting cells can be obtained by infecting dendritic cells with recombinant viruses encoding an li.MAGE-A3 fusion protein. Dendritic cells also can be loaded with HLA class I and HLA class II epitopes.

Vaccines also encompass naked DNA or RNA, encoding a MAGE-A3 HLA class II binding peptide or precursor thereof, which may be produced *in vitro* and administered via injection, particle bombardment, nasal aspiration and other methods. Vaccines of the "naked nucleic acid" type have been demonstrated to provoke an immunological response including generation of CTLs specific for the peptide encoded by the naked nucleic acid (*Science* 259:1745-1748, 1993). Vaccines also include nucleic acids packaged in a virus, liposome or other particle, including polymeric particles useful in drug delivery.

The immune response generated or enhanced by any of the treatments described herein can be monitored by various methods known in the art. For example, the presence of T cells specific for a given antigen can be detected by direct labeling of T cell receptors with soluble

fluorogenic MHC molecule tetramers which present the antigenic peptide (Altman et al., *Science* 274:94-96, 1996; Dunbar et al., *Curr. Biol.* 8:413-416, 1998). Briefly, soluble MHC class I molecules are folded *in vitro* in the presence of  $\beta$ 2-microglobulin and a peptide antigen which binds the class I molecule. After purification, the MHC/peptide complex is purified and labeled with biotin. Tetramers are formed by mixing the biotinylated peptide-MHC complex with labeled avidin (e.g. phycoerythrin) at a molar ratio of 4:1. Tetramers are then contacted with a source of CTLs such as peripheral blood or lymph node. The tetramers bind CTLs which recognize the peptide antigen/MHC class I complex. Cells bound by the tetramers can be sorted by fluorescence activated cell sorting to isolate the reactive CTLs. The isolated CTLs then can be expanded *in vitro* for use as described herein. The use of MHC class II molecules as tetramers was recently demonstrated by Crawford et al. (*Immunity* 8:675-682, 1998). Multimeric soluble MHC class II molecules were complexed with a covalently attached peptide. The class II tetramers were shown to bind with appropriated specificity and affinity to specific T cells. Thus tetramers can be used to monitor both CD4<sup>+</sup> and CD8<sup>+</sup> cell responses to vaccination protocols.

The MAGE-A3 HLA class II binding peptide, as well as complexes of MAGE-A3 HLA class II binding peptide and HLA molecule, also may be used to produce antibodies, using standard techniques well known to the art. Standard reference works setting forth the general principles of antibody production include Catty, D., Antibodies, A Practical Approach, Vol. 1, IRL Press, Washington DC (1988); Klein, J., Immunology: The Science of Cell-Non-Cell Discrimination, John Wiley and Sons, New York (1982); Kennett, R., et al., Monoclonal Antibodies, Hybridoma, A New Dimension In Biological Analyses, Plenum Press, New York (1980); Campbell, A., Monoclonal Antibody Technology, in Laboratory Techniques and Biochemistry and Molecular Biology, Vol. 13 (Burdon, R. et al. EDS.), Elsevier Amsterdam (1984); and Eisen, H.N., Microbiology, third edition, Davis, B.D. et al. EDS. (Harper & Rowe, Philadelphia (1980).

The antibodies of the present invention thus are prepared by any of a variety of methods, including administering protein, fragments of protein, cells expressing the protein or fragments thereof and an appropriate HLA class II molecule, and the like to an animal to induce polyclonal antibodies. The production of monoclonal antibodies is according to techniques well known in the art. As detailed herein, such antibodies may be used for example to identify tissues expressing protein or to purify protein. Antibodies also may be

coupled to specific labeling agents for imaging or to antitumor agents, including, but not limited to, methotrexate, radioiodinated compounds, toxins such as ricin, other cytostatic or cytolytic drugs, and so forth. Antibodies prepared according to the invention also preferably are specific for the peptide/HLA complexes described herein.

5           When “disorder” or “condition” is used herein, it refers to any pathological condition where the MAGE-3 HLA class II binding peptide is expressed. Such disorders include cancers, such as melanomas, squamous cell carcinomas of the head, neck, lung or esophagus, colorectal carcinomas, osteosarcomas, neuroblastomas, non-squamous cell carcinomas of the head or neck, ovarian tumors, lymphocytic leukemias, bladder carcinomas, prostate  
10   carcinomas, etc.

          Some therapeutic approaches based upon the disclosure are premised on inducing a response by a subject's immune system to MAGE HLA class II binding peptide presenting cells. One such approach is the administration of autologous CD4<sup>+</sup> T cells specific to the complex of MAGE-A3 HLA class II binding peptide and an HLA class II molecule to a  
15   subject with abnormal cells of the phenotype at issue. It is within the skill of the artisan to develop such CD4<sup>+</sup> T cells *in vitro*. Generally, a sample of cells taken from a subject, such as blood cells, are contacted with a cell presenting the complex and capable of provoking CD4<sup>+</sup> T lymphocytes to proliferate. The target cell can be a transfectant, such as a COS cell, or an antigen presenting cell bearing HLA class II molecules, such as dendritic cells or B cells.  
20   These transfectants present the desired complex of their surface and, when combined with a CD4<sup>+</sup> T lymphocyte of interest, stimulate its proliferation. COS cells are widely available, as are other suitable host cells. Specific production of CD4<sup>+</sup> T lymphocytes is described below. The clonally expanded autologous CD4<sup>+</sup> T lymphocytes then are administered to the subject. The CD4<sup>+</sup> T lymphocytes then stimulate the subject's immune response, thereby achieving the  
25   desired therapeutic goal.

          CTL proliferation can be increased by increasing the level of tryptophan in T cell cultures, by inhibiting enzymes which catabolizes tryptophan, such as indoleamine 2,3-dioxygenase (IDO), or by adding tryptophan to the culture (see, e.g., PCT application WO99/29310). Proliferation of T cells is enhanced by increasing the rate of proliferation  
30   and/or extending the number of divisions of the T cells in culture. In addition, increasing tryptophan in T cell cultures also enhances the lytic activity of the T cells grown in culture.

          The foregoing therapy assumes that at least some of the subject's abnormal cells

present the relevant HLA/peptide complex. This can be determined very easily, as the art is very familiar with methods for identifying cells which present a particular HLA molecule, as well as how to identify cells expressing DNA of the pertinent sequences, in this case a MAGE-A3 sequence.

5           The foregoing therapy is not the only form of therapy that is available in accordance with the invention. CD4<sup>+</sup> T lymphocytes can also be provoked *in vivo*, using a number of approaches. One approach is the use of non-proliferative cells expressing the complex. The cells used in this approach may be those that normally express the complex, such as dendritic cells or cells transfected with one or both of the genes necessary for presentation of the  
10 complex. Chen et al., (*Proc. Natl. Acad. Sci. USA* 88: 110-114, 1991) exemplifies this approach, showing the use of transfected cells expressing HPV-E7 peptides in a therapeutic regime. Various cell types may be used. Similarly, vectors carrying one or both of the genes of interest may be used. Viral or bacterial vectors are especially preferred. For example, nucleic acids which encode a MAGE-A3 HLA class II binding peptide may be operably  
15 linked to promoter and enhancer sequences which direct expression of the MAGE-A3 HLA class II binding peptide in certain tissues or cell types. The nucleic acid may be incorporated into an expression vector. Expression vectors may be unmodified extrachromosomal nucleic acids, plasmids or viral genomes constructed or modified to enable insertion of exogenous nucleic acids, such as those encoding MAGE-A3 HLA class II binding peptides. Nucleic  
20 acids encoding a MAGE-A3 HLA class II binding peptide also may be inserted into a retroviral genome, thereby facilitating integration of the nucleic acid into the genome of the target tissue or cell type. In these systems, the gene of interest is carried by a microorganism, e.g., a *Vaccinia* virus, poxviruses in general, adenovirus, herpes simplex virus, retrovirus or the bacteria BCG, and the materials de facto "infect" host cells. The cells which result present  
25 the complex of interest, and are recognized by autologous CD4<sup>+</sup> T cells, which then proliferate.

A similar effect can be achieved by combining a MAGE HLA class II binding peptide with an adjuvant to facilitate incorporation into HLA class II presenting cells *in vivo*. If larger than the HLA class II binding portion, the MAGE-A3 HLA class II binding peptide can be  
30 processed if necessary to yield the peptide partner of the HLA molecule while the TRA is presented without the need for further processing. Generally, subjects can receive an intradermal injection of an effective amount of the MAGE-A3 HLA class II binding peptide.

Initial doses can be followed by booster doses, following immunization protocols standard in the art.

A preferred method for facilitating incorporation of MAGE-A3 HLA class II binding peptides into HLA class II presenting cells is by expressing in the presenting cells a polypeptide which includes an endosomal targeting signal fused to a MAGE-A3 polypeptide which includes the class II binding peptide. Particularly preferred are MAGE-A3 fusion proteins which contain human invariant chain Ii.

Any of the foregoing compositions or protocols can include also MAGE HLA class I binding peptides for induction of a cytolytic T lymphocyte response. For example, as demonstrated below, the MAGE-A3 protein can be processed in a cell to produce both HLA class I and HLA class II responses. Several such peptides have been described in U. S. Patents 5,585,461 and 5,591,430, and PCT published application PCT/US95/03657, as well as by Gaugler et al. (*J. Exp. Med.* 179:921-930, 1994), van der Bruggen et al. (*Eur. J. Immunol.* 24:3038-3043, 1994), and Herman et al. (*Immunogenetics* 43:377-383, 1996). By administering MAGE-A3 peptides which bind HLA class I and class II molecules (or nucleic acid encoding such peptides), an improved immune response may be provided by inducing both T helper cells and T killer cells.

In addition, non-MAGE-A3 tumor associated peptides also can be administered to increase immune response via HLA class I and/or class II. It is well established that cancer cells can express more than one tumor associated gene. It is within the scope of routine experimentation for one of ordinary skill in the art to determine whether a particular subject expresses additional tumor associated genes, and then include HLA class I and/or HLA class II binding peptides derived from expression products of such genes in the foregoing MAGE-A3 compositions and vaccines.

Especially preferred are nucleic acids encoding a series of epitopes, known as "polytopes". The epitopes can be arranged in sequential or overlapping fashion (*see, e.g.,* Thomson et al., *Proc. Natl. Acad. Sci. USA* 92:5845-5849, 1995; Gilbert et al., *Nature Biotechnol.* 15:1280-1284, 1997), with or without the natural flanking sequences, and can be separated by unrelated linker sequences if desired. The polytope is processed to generate individual epitopes which are recognized by the immune system for generation of immune responses.

Thus, for example, MAGE-A3 HLA class II binding peptides can be combined with

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peptides from other tumor rejection antigens (e.g. by preparation of hybrid nucleic acids or polypeptides) and with MAGE-A3 HLA class I binding peptides (some of which are listed below) to form "polytopes". Exemplary tumor associated peptide antigens that can be administered to induce or enhance an immune response are derived from tumor associated

5 genes and encoded proteins including MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, MAGE-A12, MAGE-A13, GAGE-1, GAGE-2, GAGE-3, GAGE-4, GAGE-5, GAGE-6, GAGE-7, GAGE-8, BAGE-1, RAGE-1, LB33/MUM-1, PRAME, NAG, MAGE-Xp2 (MAGE-B2), MAGE-Xp3 (MAGE-B3), MAGE-Xp4 (MAGE-B4), tyrosinase, brain glycogen

10 phosphorylase, Melan-A, MAGE-C1, MAGE-C2, NY-ESO-1, LAGE-1, SSX-1, SSX-2(HOM-MEL-40), SSX-1, SSX-4, SSX-5, SCP-1 and CT-7. For example, antigenic peptides characteristic of tumors include those listed in Table I below.

Table I: Exemplary Antigens

	Gene	MHC	Peptide	Position	SEQ ID NO:
15	MAGE-A1	HLA-A1	EADPTGHSY	161-169	45
		HLA-Cw16	SAYGEPRKL	230-238	46
	MAGE-A3	HLA-A1	EVDPIGHL Y	168-176	47
		HLA-A2	FLWGPRALV	271-279	48
20		HLA-B44	MEVDPIGHL Y	167-176	49
	BAGE	HLA-Cw16	AARAVFLAL	2-10	50
	GAGE-1,2	HLA-Cw16	YRPRPRRY	9-16	51
	RAGE	HLA-B7	SPSSNRIRNT	11-20	52
	GnT-V	HLA-A2	VLPDVFIRC(V)	2-10/11	53,54
25	MUM-1	HLA-B44	EEKLIVVLF	exon 2/intron	55
			EEKLSVVLF (wild type)		56
	CDK4	HLA-A2	ACDPHSGHFV	23-32	57
			ARDPHSGHFV (wild type)		58
	$\beta$ -catenin	HLA-A24	SYLDSGIHF	29-37	59
30			SYLDSGIHS (wild type)		60

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5	Tyrosinase	HLA-A2	MLLAVLYCL	1-9	61
		HLA-A2	YMNGTMSQV	369-377	62
		HLA-A2	YMDGTMSQV	369-377	78
		HLA-A24	AFLPWHRLF	206-214	63
		HLA-B44	SEIWRDIDF	192-200	64
		HLA-B44	YEIWRDIDF	192-200	65
		HLA-DR4	QNILLSNAPLGPQFP	56-70	66
		HLA-DR4	DYSYLQDSDPDSFQD	448-462	67
10	Melan-A <sup>MART-1</sup>	HLA-A2	(E)AAGIGILTV	26/27-35	68,69
		HLA-A2	ILTVILGVL	32-40	70
	gp100 <sup>Pmel117</sup>	HLA-A2	KTWGQYWQV	154-162	71
		HLA-A2	ITDQVPFSV	209-217	72
15		HLA-A2	YLEPGPVTA	280-288	73
		HLA-A2	LLDGTATLRL	457-466	74
		HLA-A2	VLYRYGSFSV	476-485	75
		PRAME	HLA-A24	LYVDSLFFL	301-309
	MAGE-A6	HLA-Cw16	KISGGPRISYPL	292-303	77
	NY-ESO-1	HLA-A2	SLLMWITQCFL	157-167	79
		HLA-A2	SLLMWITQC	157-165	80
	20		HLA-A2	QLSLLMWIT	155-163

Other examples of HLA class I and HLA class II binding peptides will be known to one of ordinary skill in the art (for example, see Coulie, *Stem Cells* 13:393-403, 1995), and can be used in the invention in a like manner as those disclosed herein. One of ordinary skill in the art can prepare polypeptides comprising one or more MAGE-A3 peptides and one or more of the foregoing tumor rejection peptides, or nucleic acids encoding such polypeptides, according to standard procedures of molecular biology.

Thus polytopes are groups of two or more potentially immunogenic or immune response stimulating peptides which can be joined together in various arrangements (e.g. concatenated, overlapping). The polytope (or nucleic acid encoding the polytope) can be

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administered in a standard immunization protocol, e.g. to animals, to test the effectiveness of the polytope in stimulating, enhancing and/or provoking an immune response.

The peptides can be joined together directly or via the use of flanking sequences to form polytopes, and the use of polytopes as vaccines is well known in the art (see, e.g., Thomson et al., *Proc. Acad. Natl. Acad. Sci USA* 92(13):5845-5849, 1995; Gilbert et al., *Nature Biotechnol.* 15(12):1280-1284, 1997; Thomson et al., *J. Immunol.* 157(2):822-826, 1996; Tam et al., *J. Exp. Med.* 171(1):299-306, 1990). For example, Tam showed that polytopes consisting of both MHC class I and class II binding epitopes successfully generated antibody and protective immunity in a mouse model. Tam also demonstrated that polytopes comprising "strings" of epitopes are processed to yield individual epitopes which are presented by MHC molecules and recognized by CTLs. Thus polytopes containing various numbers and combinations of epitopes can be prepared and tested for recognition by CTLs and for efficacy in increasing an immune response.

It is known that tumors express a set of tumor antigens, of which only certain subsets may be expressed in the tumor of any given patient. Polytopes can be prepared which correspond to the different combination of epitopes representing the subset of tumor rejection antigens expressed in a particular patient. Polytopes also can be prepared to reflect a broader spectrum of tumor rejection antigens known to be expressed by a tumor type. Polytopes can be introduced to a patient in need of such treatment as polypeptide structures, or via the use of nucleic acid delivery systems known in the art (see, e.g., Allsopp et al., *Eur. J. Immunol.* 26(8):1951-1959, 1996). Adenovirus, pox virus, Ty-virus like particles, adeno-associated virus, plasmids, bacteria, etc. can be used in such delivery. One can test the polytope delivery systems in mouse models to determine efficacy of the delivery system. The systems also can be tested in human clinical trials.

As part of the immunization protocols, substances which potentiate the immune response may be administered with nucleic acid or peptide components of a cancer vaccine. Such immune response potentiating compound may be classified as either adjuvants or cytokines. Adjuvants may enhance the immunological response by providing a reservoir of antigen (extracellularly or within macrophages), activating macrophages and stimulating specific sets of lymphocytes. Adjuvants of many kinds are well known in the art; specific examples include MPL (SmithKline Beecham), a congener obtained after purification and acid hydrolysis of *Salmonella minnesota* Re 595 lipopolysaccharide, QS21 (SmithKline

Beecham), a pure QA-21 saponin purified from *Quillja saponaria* extract, DQS21, described in PCT application WO96/33739 (SmithKline Beecham), vitamin E and various water-in-oil emulsions prepared from biodegradable oils such as squalene and/or tocopherol. Cytokines are also useful in vaccination protocols as a result of lymphocyte stimulatory properties.

- 5 Many cytokines useful for such purposes will be known to one of ordinary skill in the art, including interleukin-12 (IL-12) which has been shown to enhance the protective effects of vaccines (*Science* 268: 1432-1434, 1995), GM-CSF and IL-18.

There are a number of additional immune response potentiating compounds that can be used in vaccination protocols. These include costimulatory molecules provided in either  
10 protein or nucleic acid form. Such costimulatory molecules include the B7-1 and B7-2 (CD80 and CD86 respectively) molecules which are expressed on dendritic cells (DC) and interact with the CD28 molecule expressed on the T cell. This interaction provides costimulation (signal 2) to an antigen/MHC/TCR stimulated (signal 1) T cell, increasing T cell proliferation and effector function. B7 also interacts with CTLA4 (CD152) on T cells and studies  
15 involving CTLA4 and B7 ligands indicate that the B7-CTLA4 interaction can enhance antitumor immunity and CTL proliferation (Zheng et al., *Proc. Nat'l Acad. Sci. USA* 95:6284-6289, 1998).

B7 typically is not expressed on tumor cells so they are not efficient antigen presenting cells (APCs) for T cells. Induction of B7 expression would enable the tumor cells to stimulate  
20 more efficiently CTL proliferation and effector function. A combination of B7/IL-6/IL-12 costimulation has been shown to induce IFN-gamma and a Th1 cytokine profile in the T cell population leading to further enhanced T cell activity (Gajewski et al., *J. Immunol.* 154:5637-5648, 1995). Tumor cell transfection with B7 has been discussed in relation to *in vitro* CTL expansion for adoptive transfer immunotherapy by Wang et al. (*J. Immunother.* 19:1-8, 1996).  
25 Other delivery mechanisms for the B7 molecule would include nucleic acid (naked DNA) immunization (Kim et al., *Nature Biotechnol.* 15:7:641-646, 1997) and recombinant viruses such as adeno and pox (Wendtner et al., *Gene Ther.* 4:726-735, 1997). These systems are all amenable to the construction and use of expression cassettes for the coexpression of B7 with other molecules of choice such as the antigens or fragment(s) of antigens discussed herein  
30 (including polytopes) or cytokines. These delivery systems can be used for induction of the appropriate molecules *in vitro* and for *in vivo* vaccination situations. The use of anti-CD28 antibodies to directly stimulate T cells *in vitro* and *in vivo* could also be considered.

Similarly, the inducible co-stimulatory molecule ICOS which induces T cell responses to foreign antigen could be modulated, for example, by use of anti-ICOS antibodies (Hutloff et al., *Nature* 397:263-266, 1999).

5 Lymphocyte function associated antigen-3 (LFA-3) is expressed on APCs and some tumor cells and interacts with CD2 expressed on T cells. This interaction induces T cell IL-2 and IFN-gamma production and can thus complement but not substitute, the B7/CD28 costimulatory interaction (Parra et al., *J. Immunol.*, 158:637-642, 1997; Fenton et al., *J. Immunother.*, 21:95-108, 1998).

10 Lymphocyte function associated antigen-1 (LFA-1) is expressed on leukocytes and interacts with ICAM-1 expressed on APCs and some tumor cells. This interaction induces T cell IL-2 and IFN-gamma production and can thus complement but not substitute, the B7/CD28 costimulatory interaction (Fenton et al., 1998). LFA-1 is thus a further example of a costimulatory molecule that could be provided in a vaccination protocol in the various ways discussed above for B7.

15 Complete CTL activation and effector function requires Th cell help through the interaction between the Th cell CD40L (CD40 ligand) molecule and the CD40 molecule expressed by DCs (Ridge et al., *Nature* 393:474, 1998; Bennett et al., *Nature* 393:478, 1998; Schoenberger et al., *Nature* 393:480, 1998). This mechanism of this costimulatory signal is likely to involve upregulation of B7 and associated IL-6/IL-12 production by the DC (APC).  
20 The CD40-CD40L interaction thus complements the signal 1 (antigen/MHC-TCR) and signal 2 (B7-CD28) interactions.

The use of anti-CD40 antibodies to stimulate DC cells directly, would be expected to enhance a response to tumor associated antigens which are normally encountered outside of an inflammatory context or are presented by non-professional APCs (tumor cells). Other  
25 methods for inducing maturation of dendritic cells, e.g., by increasing CD40-CD40L interaction, or by contacting DCs with CpG-containing oligodeoxynucleotides or stimulatory sugar moieties from extracellular matrix, are known in the art. In these situations Th help and B7 costimulation signals are not provided. This mechanism might be used in the context of antigen pulsed DC based therapies or in situations where Th epitopes have not been defined  
30 within known tumor associated antigen precursors.

When administered, the therapeutic compositions of the present invention are administered in pharmaceutically acceptable preparations. Such preparations may routinely

contain pharmaceutically acceptable concentrations of salt, buffering agents, preservatives, compatible carriers, supplementary immune potentiating agents such as adjuvants and cytokines and optionally other therapeutic agents.

The preparations of the invention are administered in effective amounts. An effective  
5 amount is that amount of a pharmaceutical preparation that alone, or together with further doses, stimulates the desired response. In the case of treating cancer, the desired response is inhibiting the progression of the cancer. This may involve only slowing the progression of the disease temporarily, although more preferably, it involves halting the progression of the disease permanently. In the case of inducing an immune response, the desired response is an  
10 increase in antibodies or T lymphocytes which are specific for the MAGE-A3 immunogen(s) employed. These desired responses can be monitored by routine methods or can be monitored according to diagnostic methods of the invention discussed herein.

Where it is desired to stimulate an immune response using a therapeutic composition of the invention, this may involve the stimulation of a humoral antibody response resulting in  
15 an increase in antibody titer in serum, a clonal expansion of cytotoxic lymphocytes, or some other desirable immunologic response. It is believed that doses of immunogens ranging from one nanogram/kilogram to 100 miligrams/kilogram, depending upon the mode of administration, would be effective. The preferred range is believed to be between 500 nanograms and 500 micrograms per kilogram. The absolute amount will depend upon a  
20 variety of factors, including the material selected for administration, whether the administration is in single or multiple doses, and individual patient parameters including age, physical condition, size, weight, and the stage of the disease. These factors are well known to those of ordinary skill in the art and can be addressed with no more than routine experimentation.

25

### **Examples**

We have identified antigenic peptides encoded by gene MAGE-A3 and presented to T cells in the context of HLA class II molecules. The strategy has consisted of loading dendritic cells of normal blood donors with a recombinant MAGE-A3 protein and to use these antigen-  
30 presenting cells to induce *in vitro* the activation and proliferation of specific CD4 lymphocytes. The protocol is described below (A, B, C) and in Figure 1.

**A. Processing of human blood**

Peripheral blood was obtained from the local blood bank (non cancer patients, e.g., hemochromatosis patients) as standard buffy coat preparations. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway). In order to minimize contamination of PBMC by platelets, the preparation was first centrifuged for 20 min/1000 rpm at room temperature. After removal of the top 20-25 ml, containing most of the platelets, the tubes were centrifuged for 20 min/1500 rpm at room temperature. PBMC were depleted of T cells by rosetting with 2-aminoethylisothiuronium (Sigma) treated sheep erythrocytes. The lymphocyte-depleted PBMC were left to adhere for 2 hours at 37°C in culture flasks (Falcon) at a density of  $2 \times 10^6$  cells/ml in RPMI 1640 medium supplemented with L-asparagine (0.24 mM), L-arginine (0.55 mM), L-glutamine (1.5 mM) and 1% autologous serum (complete medium). Non-adherent cells were discarded and adherent cells were cultured in the presence of IL-4 (100 U/ml) and GM-CSF (100 ng/ml) in complete medium. Cultures were fed on day 2 and 4 by removing 5 ml of the medium and adding back fresh medium with IL-4 (100 U/ml) and GM-CSF (100 ng/ml). On day 5, the non-adherent cell population was used as a source of enriched dendritic cells.

Rosetted T cells were treated with  $\text{NH}_4\text{Cl}$  (160 mM) to lyse the sheep erythrocytes, and washed.  $\text{CD4}^+$  T lymphocytes were isolated from rosetted T cells by negative selection using an anti-CD8 monoclonal antibody coupled to magnetic microbeads (Miltenyi Biotech, Germany) by sorting through the MACS magnet as recommended by the manufacturer.

**B. Cell lines, cytokines**

EBV-transformed B (EBV-B) cell lines were cultured in Iscove's modified Dulbecco medium (IMDM) (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% foetal calf serum (FCS) (Gibco BRL), 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine (AAG), 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. Human recombinant IL-2 was donated by Biogen (Geneva, Switzerland) and also was purchased from Chiron (Emeryville, CA). Human recombinant IL-4, IL-6 and IL-12 were obtained in our laboratory. Human recombinant IL-7 was purchased from Genzyme (Cambridge, MA). Human recombinant GM-CSF was donated by Sandoz (Sandoz Pharma, Basel, Switzerland) or purchased from Schering Plough (Brinny, Ireland). Human recombinant  $\text{TNF-}\alpha$  was purchased from R & D

Systems (Abingdon, UK).

**C. Feeding with protein or cell lysates and mixed lymphocyte-dendritic cells culture**

The recombinant His-MAGE-A3 protein (MAGE-A3 with a His tag) and LipoD-MAGE-A3-His protein were produced by Smith Kline Corporation Pharmaceutical Company (Rixensart, Belgium) in *E. coli* and purified by standard chromatographic procedures. LipoD-MAGE-A3-His contains one third of the lipidic form of the *Haemophilus influenzae* protein at its N-terminal residue and a polyhistidine marker at its C-terminal residue.

Autologous dendritic cells ( $5 \times 10^5$ ) were incubated at 37°C, 5% CO<sub>2</sub>, for 18-20 hours in RPMI medium supplemented with 1% autologous serum, IL-4 (100 U/ml), GM-CSF (100 ng/ml) and TNF- $\alpha$  (1 ng/ml) in the presence of the recombinant His-MAGE-A3 protein (20  $\mu$ g/ml). His-MAGE-A3 protein-pulsed dendritic cells were washed and added at  $10^4$  per round-bottomed microwell to  $10^5$  CD4<sup>+</sup> T lymphocytes in 200  $\mu$ l Iscove's medium supplemented with 10% human serum, L-asparagine (0.24 mM), L-arginine (0.55 mM), L-glutamine (1.5 mM) in the presence of IL-6 (1000 U/ml) and IL-12 (10 ng/ml). The CD4<sup>+</sup> lymphocytes were weekly restimulated with autologous dendritic cells freshly pulsed with the His-MAGE-A3 protein and were grown in complete Iscove medium supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml). Due to the short supply of dendritic cells for melanoma patient 7002, only  $6 \times 10^3$  dendritic cells were used, instead of  $10^4$ , and restimulation was performed on days 10, 20 and 30.

Dendritic cells were used for the presentation of lysates of 293-EBNA cells expressing MAGE-A3. The MAGE-A3 sequence cloned into the expression vector pCEP-4 was transiently transfected into the 293-EBNA cell line by LipofectAMINE® (GIBCO/BRL). Briefly,  $5 \times 10^4$  293-EBNA cells per flat-bottomed microwell were transfected with 6, 18, 53 or 160 ng of plasmid pCEP4-MAGE-A3 and 1  $\mu$ l of LipofectAMINE® in OptiMEM medium (GIBCO/BRL). After 24 h, transfected 293-EBNA cells were lysed in 50  $\mu$ l of complete RPMI medium by three cycles of rapid freezing-thawing. Monocyte-derived dendritic cells expressing the HLA-DR13 molecules were then added ( $1.5 \times 10^4$  cells per well) on the lysates of transfected 293-EBNA cells and maintained at 37°C for 24 hr. Dendritic cells were then washed before adding the CD4<sup>+</sup> clone. Supernatants were harvested after 20 h and assessed for IFN- $\gamma$  secretion.

**Example 1: Obtention of CD4 T cell lines and clones specific for MAGE-A3**

The microcultures that contained proliferating CD4<sup>+</sup> T cells were assessed around 35 days after the start of the culture for their capacity to produce TNF and/or IFN- $\gamma$  when stimulated with autologous EBV-B cells pulsed with the His-MAGE-A3 protein. Autologous EBV-B cells (500,000/ml) were incubated for 18-20 hours at 37°C in the presence of 20  $\mu$ g/ml of His-MAGE-A3 protein, or Ovalbumin (Sigma) as a negative control. EBV-B cells referred to herein are B cells which were immortalized with Epstein Barr virus. The EBV-B cells were prepared according to art-standard procedures. Protein-pulsed EBV-B cells were washed and added at 5,000 per round-bottomed microwell to 2,500 CD4<sup>+</sup> T lymphocytes in 150  $\mu$ l of complete Iscove's medium supplemented with L-glutamine, L-arginine, L-asparagine, 10% human serum and IL-2 (25 U/ml). After 18-20 hours, supernatants were harvested and assessed for TNF content by testing the cytotoxic effect of the supernatants on TNF-sensitive WEHI 164 clone 13 cells as previously described (Espevik and Nissen-Meyer, *J. Immunol. Methods* 95:99-105, 1986) in a MTT colorimetric assay as previously described (Hansen et al., *J. Immunol. Methods* 119:203-210, 1989; Traversari et al., *Immunogenetics* 35:145-152, 1992). IFN- $\gamma$  production was measured using an ELISA assay developed in our laboratory (see below) with reagents from Medgenix Diagnostics-Biosource (Fleurus, Belgium). Inhibition with mAbs W6/32 (anti-HLA class I) or 2B6 (anti-HLA-DR) was performed by addition of a 1/20 dilution of ascites during the experiment. Cytokine secretion in response to autologous EBV-B cells pulsed with MAGE-A3 was considered significant if it was at least twofold above the background response of T cells to EBV-B cells pulsed with ovalbumin, and if it exceeded 500 pg of IFN- $\gamma$  or 50 pg/ml of TNF per ml.

The CD4<sup>+</sup> T cell lines producing TNF specifically (Fig. 2), i.e. those which recognized the MAGE-A3 protein, were cloned by limiting dilution, using the autologous EBV-B cell line pulsed with exogenous His-MAGE-A3 protein or LipoD-MAGE-A3-His protein as stimulating cells and allogeneic EBV-B cells (LG2-EBV) as feeder cells. CD4<sup>+</sup> T cell clones were grown in complete Iscove's medium supplemented with IL-2 (50 U/ml), IL-7 (5 ng/ml) and 0.5  $\mu$ g/ml purified PHA (Murex Diagnostics, Dartford, GB). The clones were supplemented with fresh culture medium once a week and passaged with feeder cells (1.5x10<sup>6</sup> allogeneic PBL plus 5x10<sup>5</sup> LG2-EBV transformed cells per 24 well plate) at 1-2 weeks intervals. In some instances, clones were restimulated using the autologous EBV-B cell line retrotransduced with li.MAGE-A3, using 2x10<sup>5</sup> stimulating cells plus 10<sup>6</sup> LG2 EBV-B

transformed cells per 24 wells plate.

Established CD4<sup>+</sup> T cell clones were then tested for TNF and/or IFN- $\gamma$  secretion upon stimulation with autologous EBV-B cells pulsed with the exogenous His-MAGE-A3 or LipoD-MAGE-A3-His protein, as described above. Briefly, the assay was a standard ELISA in which IFN- $\gamma$  antibodies were coated onto the wells of plastic microtiter plates prior to incubation with cell supernatants to determine the amount of IFN- $\gamma$  produced. Any IFN- $\gamma$  ELISA assay could be used to measure IFN- $\gamma$  produced. Several MAGE-3 specific clones were obtained from the B6 line (Fig. 3).

The MAGE-A3 epitope is presented to the CD4 clones by HLA-DR molecules (Fig. 3): MAGE-A3 -pulsed EBV-B cells were cocultured for 24 hours at 37°C under 8% CO<sub>2</sub> with MAGE-A3 specific CD4<sup>+</sup> clones, in the continuous presence of preservative-free monoclonal antibodies used at a 1/20 dilution. Monoclonal antibody 2B6 (against HLA-DR) abolished the recognition whereas the recognition is unchanged in the presence of monoclonal antibody W6/32 (against HLA-A, B, C).

#### **Example 2: Identification of the MAGE-A3 HLA-DR restricted peptide**

In order to identify the MAGE-A3 peptides recognized by these CD4<sup>+</sup> clones, 16 amino acid peptides, corresponding to parts of the MAGE-A3 protein sequence were synthesized, loaded on the autologous EBV-B cells and tested for recognition (Fig. 4 and 5).

Peptides were synthesized using F-moc for transient NH<sub>2</sub>-terminal protection and were characterized using mass spectrometry. All peptides were >80% pure as indicated by analytical HPLC. Lyophilized synthetic peptides were dissolved in DMSO (Merck) and used at a final concentration of 500  $\mu$ M, 50  $\mu$ M or 5  $\mu$ g/ml. EBV-B cells (5,000 per round-bottomed microwell) were incubated 2 hours at 37°C, 8% CO<sub>2</sub> in the presence of the different peptides, the indicated concentrations representing the peptide concentration during the incubation step. CD4<sup>+</sup> clones were then added at 2,500 cells per well. Assay medium was Iscove's medium supplemented with L-glutamine, L-arginine, L-asparagine, 10% human serum and IL-2 (25 U/ml). After 18-20 hours, supernatants were harvested and assessed for TNF- $\alpha$  and/or IFN- $\gamma$  secretion. IFN- $\gamma$  production was measured using an ELISA test (20-4000 pg/ml) developed in the laboratory with reagents from Medgenix Diagnostics-Biosource (Fleurus, Belgium).

In one set of experiments, the peptides were screened at a non-physiologic

concentration of 500  $\mu$ M. Non-physiologic concentrations of peptide may lead to non-specific activation of T cells clones. Indeed, when used at 500  $\mu$ M, peptide MAGE-A3<sub>159-174</sub> (Fig. 4-peptide 335; SEQ ID NO:6) induced activation of clones 436/B6.34 (B6/34) and 436/B6.37 (B6/37), but this peptide was not effective in activating these clones when used at 50  $\mu$ M (Fig. 5). On the contrary, the peptides RKVAELVHFLLLKYRA (MAGE-A3<sub>111-126</sub> Fig. 4-peptide 323; SEQ ID NO:3) and ELVHFLLLKYRAREPV (MAGE-A3<sub>115-130</sub> Fig. 4-peptide 324; SEQ ID NO:4) stimulated specifically TNF- $\alpha$  and IFN- $\gamma$  production by clones B6/34 and B6/37 when used at more physiologic concentrations or preferably lower concentrations. These two peptides were also able to induce the proliferation of the B6 clones (Fig. 6).

As shown in Figs. 3-6, clones F3/40 and F3/37 did not recognize MAGE-A3 peptides, did recognize cells presenting MAGE-A3. Thus it is believed that these two clones recognize a cellular component rather than MAGE-A3.

### Example 3: Determination of the HLA restriction element utilized by MAGE-A3 specific CD4<sup>+</sup> clone B6/37

Cytokine secretion by these CD4<sup>+</sup> clones in response to autologous EBV-B cell pulsed with the His-MAGE-A3 protein is restricted to HLA-DR. To further define the HLA-restriction element utilized by clone B6/37, additional EBV-B cell lines were used for peptide presentation as described above (Fig. 7). HLA serotyping of AUMA-EBV (LB1622-EBV), LB 1555-EBV, GERL-EBV (MZ2-EBV) revealed that class II molecules shared by all three cell types were limited to HLA-DRB1/1302. Moreover, ADET-EBV (LB1118-EBV) was found to present effectively the MAGE-A3<sub>115-130</sub> peptide and the HLA serotyping of these cells was found to be HLA-DRB1/1301. Screening of several other EBV-B cell lines as described above for their ability to stimulate clone B6/37 when pulsed with peptide MAGE-A3<sub>111-126</sub> and MAGE-A3<sub>115-130</sub> was performed to confirm that both HLA-DRB1/1301 and HLA-DRB1/1302 can present these peptides (Table II).

**Table II**

EBV-B cell line	IFN- $\gamma$ production (pg/ml) by clone B6/37 stimulated by peptide	
	MAGE-A3 <sub>111-126</sub>	MAGE-A3 <sub>115-130</sub>

- 43 -

	<i>DR13 positive</i>		
	LB1118	3761	3909
	LB1555	>4000	>4000
	LB1622	1731	1349
5	MZ2	3429	3096
	OMW	>4000	>4000
	<i>DR13 negative</i>		
	BM16	99	120
10	BOB	128	101
	BOLETH	77	67
	LKT3	6	27
	OLGA	117	68
	RML	134	129
15	RSH	140	87
	TAB089	52	55
	TISI	119	179
	VAF	62	178
20	VET	105	135

Screening of other EBV-B cell lines as described above for their ability to stimulate clones B6/34 and/or B6/37 when pulsed with peptide peptide MAGE-A3<sub>111-126</sub> and/or MAGE-3<sub>115-130</sub> is performed to define other HLA-DRB1/13 presenting molecules.

25

#### **Example 4: Determination of minimal peptides still able to stimulate B6/37 clone**

Unlike HLA-class I-restricted peptides, class II-restricted peptides vary considerably in length and can tolerate extensions at both the amino and carboxy termini. It was demonstrated that both peptides MAGE-A3<sub>111-126</sub> and MAGE-A3<sub>115-130</sub> stimulated specifically clones B6/34 and B6/37, whereas peptides MAGE-A3<sub>107-122</sub> and MAGE-A3<sub>119-134</sub> were unable to activate these clones. Therefore, the MAGE-A3<sub>115-126</sub> peptide (ELVHFLLLKYRA; SEQ ID

30

NO:9) may be the minimal 12 amino-acids motif necessary for activation of B6/34 and B6/37 clones. As expected, peptide MAGE-A3<sub>115-126</sub> induced significant production of IFN- $\gamma$  by clone B6/37 (Fig. 8). Shortened peptides having deletions of one residue or more also were prepared. Several of the shortened peptides, e.g. MAGE-A3<sub>116-126</sub> (SEQ ID NO:10) and  
5 MAGE-A3<sub>117-126</sub> (SEQ ID NO:11), also induced IFN- $\gamma$  production by clone B6/37 (Fig. 8), albeit reduced amounts of IFN- $\gamma$ . MAGE-A3<sub>118-126</sub> (SEQ ID NO:12) did not induce the production of significant amounts of IFN- $\gamma$ .

#### **Example 5: Presentation of exogenous MAGE-A3 by dendritic cells**

10 Clone 37 was obtained with autologous dendritic cells heavily loaded with MAGE-A3 protein. To determine the ability of dendritic cells that capture a quantity of MAGE-A3 protein released by a few MAGE-A3<sup>+</sup> cells to stimulate CD4<sup>+</sup> T cells, HLA-DR13 dendritic cells were incubated for 24h with decreasing concentrations of MAGE-A3 as depicted in (Fig. 9A). Stimulation of clone 37 was tested by washing the cells, distributing the cells in round-  
15 bottomed microwells, and adding 2500 clone 37 cells. IFN- $\gamma$  secretion was measured by ELISA after 20h of coculture. Experiments were performed in triplicate. Half maximum production of IFN- $\gamma$  was obtained when 10<sup>4</sup> dendritic cells in a volume of 100  $\mu$ l were pre-incubated with 30 ng of MAGE-A3, the quantity of MAGE-A3 present in around 2 x 10<sup>4</sup> MZ2-MEL.43 tumor cells. This result suggests that dendritic cells which endocytose debris  
20 of very few tumor cells expressing MAGE-A3 are able to stimulate clone 37.

Another assay was performed to test the ability of dendritic cells to process the debris of MAGE-A3<sup>+</sup> cells: MAGE-A3 was transiently transfected into 293-EBNA cells, which were subsequently lysed. HLA-DR13 dendritic cells were first incubated for 24 h with lysates and then used to stimulate clone 37. In three separate experiments, clone 37 was stimulated by  
25 dendritic cells incubated with lysates of 293-EBNA cells transfected with pCEP4-MAGE-A3 as judged by IFN- $\gamma$  release (Fig. 9B). 293-EBNA cells (5 X 10<sup>5</sup> cells per well) were transfected with different doses of pCEP4-MAGE-A3 mixed with lipofectAMIN<sup>®</sup>, and lysed 24 h after transfection. HLA-DR13 dendritic cells were cultured 24 h with 293-EBNA lysate (the equivalent of 5 cells per dendritic cell). Dendritic cells were then washed, distributed in  
30 round-bottomed microwells, and 2500 cells of clone 37 were added. IFN- $\gamma$  secretion was measured by ELISA after 20 h of coculture. The experiment was performed in triplicate.

**Example 6: Preparation and use of MAGE-A3 fusion proteins**

The MAGE-A3 protein was expressed in an EBV B cell line MZ2 EBV (HLA A1 DR13) as a fusion protein with a truncated invariant chain (Ii) or with the lysosome-associated membrane protein (LAMP-1) to target the presentation of MAGE-A3 derived peptides in HLA class II molecules (see schematic drawing in Fig.14). Transduction of Ii MAGE-A3 yielded peptide presentation in HLA class II, as measured by the recognition by the CD4 T cell clone LB 1555 CD4 436/B6.37, which reacts with the MAGE-A3.DR13 epitope. In addition, expression of Ii MAGE-A3 in EBV B cells resulted in peptide presentation in HLA class I, which was determined by the activation of the MAGE-A3.A1 specific CTL clone LB 705 434/1. In contrast, expression of the MAGE-A3-LAMP-1 fusion protein only marginally enhanced the presentation of MAGE-A3 peptide in HLA molecules. Connecting the Ii to MAGE-A3 therefore can be used as a vaccine to induce presentation of MAGE-A3-derived peptides in both HLA class I and class II.

**Plasmids and cloning of fusion constructs**

*MAGE-A3*: The MAGE-A3 cDNA and polypeptide are set forth as SEQ ID NO:1 and SEQ ID NO:2, respectively.

*Human invariant chain*: The plasmid named IipSV51L containing the human invariant chain encoding cDNA was kindly provided by Dr. J. Pieters (Basel Institute for Immunology, Basel, Switzerland; *J. Cell Science* 106:831-846, 1993).

*LAMP1*: The plasmid pCMV-sig E7-LAMP1 was kindly provided by Dr. T. Wu (Johns Hopkins University, Baltimore, MD, USA; *Proc. Natl. Acad. Sci. USA* 92:11671-11675, 1995).

*pMFG*: The plasmid pMFG was kindly provided by Dr. O. Danos (Somatix Therapy Corporation, Alameda, CA, USA).

**Construction of pMFG-MAGE-A3:**

The MAGE-A3 cDNA was transferred to the pMFG vector after the introduction of the appropriate restriction enzyme recognition sites at the 5' and 3' end of the coding sequence. A *NcoI* site was introduced at the 5' site and a *BglII* site at the 3' end by PCR using the primers: *NcoI*-sense: 5'-TTTCCATGGCTCTTGAGCAGAGGAGTCAGC-3' (SEQ ID NO:14) and *BglII*-antisense: 5'-CCCAGATCTTCACTCTTCCCCCTCTCTC-3' (SEQ ID

NO:15) [the recognition sites for *NcoI* and *BglII* are in italics]. The PCR product was cloned into a pCR2.1 and sequenced according to standard methods. The *NcoI*-*BglII* amplification product was cloned into pMFG opened with the enzymes *NcoI* and *BamHI*.

5     Construction of pMFG-Ii.MAGE-A3

The cDNA encoding the amino terminal end (i.e. the cytoplasmic tail and the transmembrane region) of the human invariant chain polypeptide (hu-Ii; residues 1 - 80) was amplified by PCR using IipSV51L as template. The following primers were used: hu-Ii sense: 5'-TTTCCATGGATGACCAGCGCGAC-3' (SEQ ID NO:16); and hu-Ii antisense: 5'-  
10    TTTGGATCCGGAAGCTTCATGCGCAGGTTC-3' (SEQ ID NO:17) [the recognition sites for *NcoI* and *BamHI* are in italic]. The PCR product was cloned into pCR2.1 and sequenced according to standard methods. The *NcoI*-*BamHI* amplification product was cloned into pMFG, opened with the enzymes *NcoI* and *BamHI* resulting in pMFG-Ii.

A *BglII* recognition site, replacing the ATG codon and in frame with the *BamHI* site at  
15    the 3' end of the truncated Ii-cDNA, was introduced at the 5' end of the MAGE-A3 cDNA by PCR using the primers: *BglII*-sense: 5' TTTAGATCCTGAGCAGAGGAGTCAGC-3' (SEQ ID NO:18) and *BglII*-antisense (SEQ ID NO:15) [the recognition sites for *BglII* are in italic]. The PCR product (*BglII*.MAGE-A3.*BglII*) was cloned into pCR2.1 and sequenced according to standard methods.

20    The recombinant plasmid pMFG-Ii was reopened with *BamHI* and the *BglII*.MAGE-3.*BglII* amplification product was ligated to the compatible ends. Recombinant plasmids containing the MAGE-A3 cDNA in frame and in the right orientation were identified by restriction fragment analysis.

25    Construction of pMFG-Sig.MAGE-A3.LAMP-1

The cDNAs encoding the signal peptide of the LAMP-1 protein and the transmembrane domain and cytoplasmic tail of LAMP-1 were amplified by PCR using pCMV-sig E7-LAMP1 as template. The primer set for the signal peptide of LAMP-1 was: Sig sense: 5'-CCCCCATGGCGGCCCCCGGC-3' (SEQ ID NO:19) and Sig antisense:  
30    5'-GGGGGATCCTCAAAGAGTGCTGA-3' (SEQ ID NO:20) [the recognition sites for *NcoI* and *BamHI* are in italic]. The *BamHI* site at the 3' end of this cDNA is in frame with the *BglII* site at the 5' end of the *BglII*.MAGE-A3.*BglII* fragment. The amplification product was

cloned into pMFG to prepare pMFG-Sig.

The primer set for the amplification of the LAMP1 transmembrane domain and cytoplasmic tail was: LAMP-1 sense: 5'-GGGGGATCCTAACAACATGTTGATCCCC-3' (SEQ ID NO:21) and LAMP-1 antisense: 5'-GGGAGATCTCTAGATGGTCTGGGTCTGA

5 TAGCCGGC-3' (SEQ ID NO:22) [the recognition sites for *Bam*HI and *Bgl*II are in italic]. The amplification product was sequenced according to standard methods and cloned into pMFG-Sig, resulting in plasmid pMFG-Sig.LAMP-1 with an unique *Bam*HI site at the junction of the signal peptide and the transmembrane sequence. To generate the plasmid pMFG-Sig.MAGE-A3.LAMP-1, a *Bgl*II - *Bam*HI fragment isolated from the *Bgl*II.MAGE-A3.*Bgl*II cDNA was cloned into pMFG-Sig.LAMP-1 opened with *Bam*HI. This cloning step

10 deleted the 3' end of MAGE-A3 encoding amino acids 240 - 314.

#### Cell lines, media and reagents

The PhoenixAMPHO cell line (kindly provided by Dr. Nolan, Stanford University

15 School of Medicine, CA, USA) is a high titer amphotropic retrovirus producing cell line that has been generated by stable transfection of 293T cells with a Moloney GagPol-IRES-Lyt 2 construct with an RSV promoter and a pPGK hygro selectable marker. These cells were then stably transfected with the Moloney amphotropic envelope gene driven by a CMV promoter and co-selected with the diphtheria toxin resistance gene (pHED-7). This producer cell line is

20 helper virus free.

PhoenixAMPHO cells were cultured and passaged in 175 cm<sup>2</sup> flasks in DMEM (Life Technologies, Ghent, Belgium) supplemented with 10% heat inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

The MZ2-EBV B cell line was generated from B cells of melanoma patient MZ2

25 (HLA A1 A29 DR0101 DR1302) by infection with EBV. Likewise, the LG2-EBV B cell line was generated from non-cancer patient LG2 (HLA A24 A32 DR7 DR14). MZ2-MEL.43 is a melanoma cell line from patient MZ2. The EBV transformed B cell lines and MZ2-MEL.43 were cultured in Iscove's modified Dulbecco's (ID) medium supplemented with 10% foetal calf serum (FCS), 0.24 mM L-asparagine, 0.55 mM L-arginine and 1.5 mM L-glutamine

30 (AAG).

The cytotoxic T cell clone LB 705 CTL 434/1 is directed against the MAGE-A3.A1 epitope and was generated in a primary culture of CD8<sup>+</sup> T cells from non-cancer patient

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LB705 (HLA A1 A2) and irradiated autologous PBL (peripheral blood lymphocytes) pulsed with the MAGE-A3.A1 peptide. The CD4 T cell clone LB 1555 CD4 436/B6.37 recognized the MAGE-A3.DR13 epitope and was identified by a primary culture of T cells of patient LB 1555 DESA (HLA DR3 DR1302) and autologous monocyte-derived dendritic cells  
5 preincubated with purified MAGE-A3 protein. The T cell clones were cultured in ID supplemented with 10% heat-inactivated human serum (HS), AAG and 50 U/ml recombinant human IL-2 (rh IL-2) in the presence of irradiated feeder cells (LG2-EBV, pooled human PBL) and specific stimulating cells (MZ2-MEL-43 for CTL 434/1 or DESA-EBV preincubated with the MAGE-A3 protein for the CD4 T cell clone 436/B6.37).

10

#### Generation of high titer MAGE 3 encoding recombinant retrovirus

The MAGE-A3 encoding retroviral vector plasmids, MFG-MAGE-A3, MFG-  
11. MAGE-A3, MFG-Sig.MAGE-A3.LAMP and MFG-EGFP (encoding enhanced green  
fluorescent protein reporter), were introduced into the PhoenixAMPHO packaging cells by  
15 transfection. The MFG retroviral vector is derived from Moloney murine leukemia virus and is lacking in a drug resistance marker nor does it express any other potential antigenic protein except for the inserted cDNA (Rivière, *Proc. Natl Acad. Sci. USA* 92:6733-6737, 1995). The transfection procedure is a modification of the calcium phosphate-mediated transfection protocol of Graham and van der Eb (*Virology* 54:536-539).

20

Twenty four hours prior to transfection,  $10.8 \times 10^6$  PhoenixAMPHO cells were plated in 14 ml cell growth medium in a 75 cm<sup>2</sup> tissue culture flask (Falcon). After adding the cells, the flask was gently shaken forward and backward to distribute cells evenly about the flask bottom. The cells were incubated at 37°C and 5% CO<sub>2</sub>. At the time of transfection, when the cells should have reached a confluence of 70-80%, the medium was removed and was  
25 replaced by 14 ml fresh PhoenixAMPHO cell growth medium containing 25 mM chloroquine (Sigma Chemical Co., St. Louis, MO, USA). A transfection cocktail was prepared in a 50 ml tube by adding 40 µg retroviral vector plasmid DNA to water and diluting to 1575 µl final volume. To this DNA solution 225 µl of 2 M CaCl<sub>2</sub> (Sigma) was added. Then, 1800 µl of 2x HeBS (50mM HEPES, 10 mM KCl, 12 mM dextrose, 280 mM NaCl and 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>  
30 dissolved in distilled water, filtered through 0.2 µ filter and stored at -20°C) was added dropwise to the DNA/CaCl<sub>2</sub> solution by vigorously bubbling for 15 seconds with an automatic pipette. The DNA/CaCl<sub>2</sub>/HeBS mix was added immediately and dropwise onto the cells and

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the flask was gently swirled to ensure uniform mixing of DNA/CaPO<sub>4</sub> particles. The cells were incubated at 37°C / 5% CO<sub>2</sub> for 7 to 9 hours and the chloroquine containing medium was changed for fresh PhoenixAMPHO cell growth medium. Approximately 24 hours prior to the harvest of the retroviral supernatant, the PhoenixAMPHO medium was removed and  
5 gently replaced by 9 ml of EBV cell growth medium (Iscove's) containing only 2.5% FCS. The retroviral supernatant was harvested 48 hours following transfection by removing the medium from the cells and filtering through a 0.45 µ filter to remove cell debris. After harvest and filtration, the virus containing medium was kept on ice, aliquoted in appropriate volumes in 15 ml polypropylene tubes and stored at -80°C. The MFG-EGFP transfected  
10 PhoenixAMPHO cells were assayed for transfection efficiency by FACS analysis.

#### Retroviral transduction of EBV cell lines

The EBV transformed cells were infected by resuspending the cells in an infection cocktail and centrifugation. Target cells were resuspended in 60 mm tissue culture plates  
15 (Falcon) at a density of  $1.0 \times 10^6$  cells in 4 ml infection cocktail. The plates were centrifuged for 2 hours at 32°C and 1200 rcf in an IEC centrifuge, rotor type 228. For each plate to be transduced, 4 ml of injection cocktail was prepared by diluting the viral supernatant 1:2 in EBV cell growth medium and adding protamine sulfate to a final concentration of 6 µg/ml. Centrifugation was followed by another 2 hours of incubation in a humidified incubator at  
20 37°C and cells were transferred to 4 ml of target cell growth medium. This transduction cycle was carried out immediately after plating the cells and was repeated at 24 and 48 hours. The infected EBV cells were assayed for EGFP reporter gene expression by FACS analysis 24 to 48 hours following the third infection cycle.

#### Interferon-γ production assay.

5000 T cells of LB705 CTL 434/1 or 3000 T cells of clone LB1555 CD4 436/B6.37 were washed and cultured overnight in the presence of 5000 retrovirally transduced EBV B cells of MZ2-EBV, or LG2-EBV B cells, in 100 µl ID medium containing 10% HS, AAG and 50 U/ml rh IL-2 in a round-bottom 96 wells plate. All cocultures were performed in triplicate.  
30 50 µl culture supernatant was assayed for the presence of IFN-γ by ELISA (IFN-γ ELISA, Biosource). Briefly, ELISA plates precoated with anti-human IFN-γ Ab were washed and incubated with 50 µl culture supernatant and 50 µl biotinylated anti-human IFN-γ Ab (1:1250

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in ID, 10% HS, AAG) for 2 h at room temperature (RT). After three washings the plates were incubated with 50  $\mu$ l per well horseradish peroxidase conjugated streptavidin (1:3000 in PBS/0.5% BSA) for 30 min at RT, which was detected by TMB substrate, and H<sub>2</sub>SO<sub>4</sub> to stop the reaction. The optical density was read at 450 nm. Samples containing 4000 pg/ml IFN- $\gamma$  and 1:2 dilutions were used as standards.

#### Cytotoxicity assay.

1x10<sup>6</sup> EBV B cells were labeled with 25  $\mu$ Ci Na<sub>2</sub><sup>51</sup>CrO<sub>4</sub> for 60-90 min at 37°C. The cells were washed and resuspended at 1 x 10<sup>4</sup>/ml. In control assays, MAGE-A3.A1 peptide was added to the cell suspension at a concentration of 1  $\mu$ M. LB705 CTL 434/1 T cells were cultured with 1000 labeled target cells per well in V shaped 96 well plates at 37°C at effector to target cell ratios of 30 to 1 and ten-fold dilutions. After 4h, the chromium release (ER) was measured in an aliquot of 100  $\mu$ l supernatant. Target cells incubated in medium only or in 1% Triton were taken as minimal (SR) and maximal (MR) <sup>51</sup>Cr release, respectively. The percentage experimental <sup>51</sup>Cr release in the samples was calculated as: (ER-SR/MR-SR) x 100%.

#### Recognition of transduced EBV B cell lines by T cell clone LB1555 CD4 436/B6.37.

MZ2 EBV were transduced with MFG Ii MAGE-A3 (MZ2 EBV-Ii MAGE-A3), MFG SigMAGE-A3 LAMP (MZ2 EBV-SigMAGE-A3 LAMP), MFG MAGE-A3 (MZ2 EBV-MAGE-A3) or with MFG EGFP (MZ2 EBV-EGFP). Transduced cells were cultured overnight in the presence of the CD4<sup>+</sup> T cell clone LB1555 CD4 436/B6.37, which reacts with the MAGE-3.DR13 epitope. The T cell clone recognized MZ2-EBV-Ii MAGE-A3, as determined by the release of IFN- $\gamma$  in culture supernatant measured by ELISA (Fig. 10). In contrast, M2 EBV-SigMAGE-A3 LAMP only induced a weak production of IFN- $\gamma$ . The control transfectants, MZ2 EBV-MAGE-A3 and MZ2 EBV-EGFP, and LG2 EBV (not shown) were not recognized by the CD4 T cells. These results show that the Ii-MAGE-A3 fusion protein is processed for presentation by HLA class II, whereas the MAGE-A3 protein alone does not reach the HLA class II antigen presentation pathway.

#### Recognition of transduced EBV B cell lines by LB 705 CTL 434/1.

Both MZ2 EBV-Ii MAGE-A3 and MZ2 EBV-SigMAGE-A3 LAMP were recognized

to the same extent by the MAGE-A3.A1 specific CTL clone LB 705 CTL 434/1 after overnight coculture (Fig. 11). IFN- $\gamma$  release in the culture supernatant was measured by ELISA. MZ2 EBV-Ii MAGE-A3 elicited a high IFN- $\gamma$  production by the CTL clone, indicating that expression of the MAGE-A3 protein fused to the Ii can still lead to processing in the HLA class I pathway.

The lysis by LB705 CTL 434/1 of MZ2-EBV retrovirally transduced with MAGE-A3, Ii MAGE-A3, SigMAGE-A3 LAMP or GFP was tested in a 4h  $^{51}\text{Cr}$  release assay at various effector to target cell (E/T) ratios. In parallel, MAGE-A3.A1 peptide was added as a positive control for T cell activation. MZ2 EBV-Ii MAGE-A3 and MZ2 EBV-SigMAGE-A3 LAMP were both lysed by the LB705 CTL 434/1, similar to MZ2 EBV-MAGE-A3. The percentage of lysis was equal to the target cell lysis in the presence of the MAGE-A3.A1 peptide (Fig. 12).

#### Presentation of MAGE-A3 derived peptide in HLA class II by MZ2-MEL.43.

To further confirm to contribution of the Ii to presentation of MAGE-A3 peptides in HLA class II molecules, the melanoma cell line MZ2-MEL.43 was transduced with MFG Ii MAGE-A3 (MZ2-MEL.43-Ii MAGE-A3) or with MFG EGFP (MZ2-MEL.43-EGFP). One month after transduction with MFG EGFP, 90% of MZ2-MEL.43 cells expressed high levels of EGFP (not shown). MZ2-MEL.43 expresses the MAGE-A3 protein endogenously, but does not present MAGE-A3-derived peptides in HLA class II. However, after transduction of MZ2-MEL.43 with MFG-Ii MAGE-A3, it was recognized by the CD4 T cell clone LB1555 CD4 436/B6.37 after overnight coculture (Fig. 13A). IFN- $\gamma$  release in the culture supernatant was measured by ELISA. This indicates that in contrast to the endogenously expressed MAGE-A3, the Ii MAGE-A3 can be processed for presentation in HLA class II. Both parental and transduced MZ2-MEL.43 activated the CTL clone LB705 CTL 434/1 (Fig. 13B).

#### **Example 7: Two other CD4<sup>+</sup> clones recognized a second MAGE-A3 epitope**

In order to confirm the efficacy of this protocol, dendritic cells were fed with the MAGE-3 protein, using conditions described above, to stimulate autologous CD4<sup>+</sup> T lymphocytes from hemochromatosis patient LB1158 or from melanoma patient 7002. After four restimulations, the specific production of IFN- $\gamma$  by an aliquot of each of the microcultures stimulated with the autologous EBV-B cells loaded with MAGE-A3 were tested

on day 35 for patient LB1158 and day 37 for patient 7002. For each individual, one positive microculture was cloned using autologous EBV-B cells as stimulating cells, either retrotransduced with MFG Ii MAGE-A3 for LB1158, or fed with protein MAGE-A3 for patient 7002. LB1158 CD4<sup>+</sup> clone 22 and 7002 CD4<sup>+</sup> clone 2 were obtained. Both clones  
 5 secreted IFN- $\gamma$  upon stimulation with autologous EBV-B cells loaded with MAGE-A3, or transduced with MFG Ii MAGE-A3, which proves that the clones are specific for MAGE-A3 and are not directed against a contaminant in the batch of protein. For clone 22, IFN- $\gamma$  release was inhibited by mAb 2B6 which recognizes HLA-DR molecules. Specifically, autologous EBV-B cells were incubated for 20 h in the presence of 20  $\mu$ g/ml of recombinant His-MAGE-  
 10 A3 protein. Clone 22 (2500 cells) was incubated overnight with 5000 MAGE-A3 loaded EBV-B cells in round bottom microwells in the presence or absence of monoclonal antibodies which recognize HLA-A,B,C (W6/32) or HLA-DR (2B6). IFN- $\gamma$  secretion was measured after 20 h by ELISA (Fig. 15A).

Peptides corresponding to parts of MAGE-A3 (e.g., MAGE<sub>107-122</sub>, MAGE<sub>111-126</sub>,  
 15 MAGE<sub>115-130</sub>, MAGE<sub>119-134</sub>) were tested for recognition by clone 2 and 22. HLA-DR13 EBV-B cells were pulsed for 2 h with different concentrations of these peptides. Clones 2 and 37 (2500 cells) were incubated with 5000 peptide pulsed cells in round-bottomed microwells for 20 h and assayed for IFN- $\gamma$  production. Peptide MAGE-A3<sub>119-134</sub> (FLLKRYRAREPVTKE; SEQ ID NO:23) scored positive. It stimulated secretion of IFN- $\gamma$  by clone 2 at a  
 20 concentration of 10 ng/ml, whereas clone 37 was not stimulated by this peptide in the same conditions (Fig. 15B). Clone 22 was also stimulated by peptide MAGE-A3<sub>119-134</sub> (SEQ ID NO:23) in similar experiments.

Donor LB1158 expresses the DRB1\*0101, DRB1\*1301 and DRB3\*0202 alleles, whereas patient 7002 expresses the DRB1\*0101, DRB1\*1302 and DRB3\*0301. A number of  
 25 additional EBV-B cell lines were tested, which revealed that only those expressing DR13 were able to present the peptide to clone 2 and 22 (Table III). Autologous or allogeneic EBV-B cells were pulsed for 2 h with 1  $\mu$ g/ml MAGE-A3<sub>119-134</sub> peptide, and then washed. Clones 2 or 22 were incubated with 5000 peptide-pulsed EBV-B cells in round-bottomed microwells for 20 h and the clones' reactivity to the EBV-B cells was measured by ELISA as IFN- $\gamma$   
 30 secretion. Experiments were performed in triplicate.

Table III

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		EBV-B cell line		IFN- $\gamma$ production (pg/ml) after stimulation with peptide MAGE-A3 <sub>119-134</sub> of	
				clone 22	clone 2
5		<i>DR13 positive</i>			
		7002		>4000	>4000
		LB1118		1345	>4000
		LB1158		3967	>4000
10		LB1622		1250	3993
		LB1859		>4000	>4000
		LB1861		3865	>4000
		LB1869		>4000	>4000
		MZ2		2030	>4000
15		OMW		3651	3153
		<i>DR13 negative</i>			
		BM16		7	12
		BOB		46	59
20		BOLETH		6	0
		LKT3		0	0
		OLGA		0	0
		RML		6	0
		RSH		0	0
25		TAB089		0	0
		TISI		4	4
		VAF		49	0
		VET		56	0

30

**Example 8: Definition of minimal MAGE-A3 antigenic peptides**

Unlike HLA-class I-restricted peptides, class II-restricted peptides usually vary in length and tolerate extensions at both the amino and carboxy termini, because they are not fixed by their ends in the groove. It is therefore difficult to define precisely the length of the antigenic peptide. Therefore a large number of MAGE-A3 peptides were tested at different concentrations (Tables IV and V). As shown in Table IV, autologous LB1555 EBV-B cells were incubated for 2 h with different MAGE-A3 peptides. 2500 cells of clone 37 were then cocultured for 20 h in round-bottomed microwells with 5000 peptide-pulsed cells. IFN- $\gamma$  released in the supernatant of the cocultures was measured in an ELISA assay. The experiments were performed in triplicate. As shown in Table V, HLA-DR13 EBV-B cells were incubated for 2 h with different MAGE-A3 peptides. Clones 2 and 22 (2500 cells) were then cocultured for 20 h in round-bottomed microwells with 5000 peptide-pulsed cells. IFN- $\gamma$  released in the supernatant of the cocultures was measured in an ELISA assay. The experiments was performed twice in duplicates. From these experiments it was concluded that a short peptide well recognized by clone 37 was AELVHFLLLKYRAR (MAGE-A3<sub>114-127</sub>; SEQ ID NO:28) whereas short peptides well recognized by clone 2 and 22 were FLLLLKYRAREPVT (MAGE-A3<sub>119-131</sub>; SEQ ID NO:34) and LLKYRAREPVTKAE (MAGE-A3<sub>121-134</sub>; SEQ ID NO:38).

As for HLA-class I-restricted peptides, HLA-class II-restricted peptides have preferred anchor residues within a HLA binding core of 9 to 10 amino acids. Most peptides binding to HLA-DRB1\*1301 and B1\*1302 are characterized by an I, L or V at the P1 position in the binding core; a L, V, M, A, W or Y at the P4 position; a R or K at the P6 position and a Y, F, A, S or T at the P9 or 10 position (Rammensee et al., MHC Ligands and Peptide Motifs, Molecular Biology Intelligence Unit, Springer, 1997). The foregoing results suggest that for the first antigenic peptide (recognized by clone 37), the 10 amino acids peptide LVHFLLLKYR (MAGE-A3<sub>116-125</sub>; SEQ ID NO:41) is the binding core for HLA-DR13, L116 and Y125 residues serving respectively as P1 and P9/10 anchors (Table IV). Alternatively, for clone 37 recognition the 10 amino acid peptide VHFLLLKYRA (MAGE-A3<sub>117-126</sub>; SEQ ID NO:11) can be the binding core for HLA-DR13, with V117 and A126 serving respectively as P1 and P9 anchors. For the second antigenic peptide (recognized by clone 2 and 22), the results suggest that the 10 amino acids peptide LKYRAREPVT (MAGE-A3<sub>122-131</sub>; SEQ ID NO:42) is the binding core for HLA-DR13, with residues L122, R127, and T131 serving respectively as P1, P6 and P10 anchors (Table V). Thus the peptides described herein for

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binding clones 37, 2 or 22 are encoded by nucleic acids which include the nucleotide sequences GTTCATTTTC TGCTCCTCAA GTATCGAGCC (SEQ ID NO:13) or TTGGTTCATT TTCTGCTCCT CAAGTATCGA (SEQ ID NO:43) for clone 37, and CTCAAGTATC GAGCCAGGGA GCCGGTCACA (SEQ ID NO:44), for clones 2 or 22.

5

**Table IV**

		peptide concentration (ng/ml)						
		1000	300	100	30	10	3	1
		IFN- $\gamma$ production (pg/ml) by clone 37						
10	VAELVHFLLLKYRARE	>4000	>4000	>4000	1647	376	300	30
	VAELVHFLLLKYRAR	>4000	>4000	>4000	2963	779	262	146
	VAELVHFLLLKYRA	>4000	>4000	2534	648	87	179	7
	AELVHFLLLKYRARE	>4000	>4000	3837	1473	235	230	38
	AELVHFLLLKYRAR	>4000	>4000	>4000	2862	802	272	151
15	AELVHFLLLKYRA	>4000	>4000	>4000	2523	620	139	134
	ELVHFLLLKYRARE	>4000	>4000	3095	1087	166	160	25
	ELVHFLLLKYRAR	>4000	>4000	3633	1899	333	192	47

20

**Table V**

		Peptide concentration (ng/ml)						
		1000	300	100	30	10	3	1
		IFN- $\gamma$ production (pg/ml) by clone 22						
25	FLLLK YRAREPVTKA	2180	1965	1854	1775	1619	1294	283
	FLLLK YRAREPVTK	453	115	28	10	1	0	0
	FLLLK YRAREPVT	2183	1834	1510	1635	1099	584	291
	FLLLK YRAREPV	1353	746	383	132	13	3	0
	FLLLK YRAREP	30	7	6	7	1	0	0
30	LLLK YRAREPVTKAE	2214	1739	1679	1832	1440	965	718

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LLKYRAREPVTKAE	2225	1826	1736	1651	1073	638	262
LKYRAREPVTKAE	2004	1475	1029	567	223	25	0
KYRAREPVTKAE	542	135	16	5	0	5	0

5	IFN- $\gamma$ production (pg/ml) by clone 2						
	FLLLLKYRAREPVTKA	3314	3354	2871	2713	1753	865 245
	FLLLLKYRAREPVTK	0	0	85	0	0	0 0
	FLLLLKYRAREPVT	3079	2860	2951	2003	1437	370 114
	FLLLLKYRAREPV	2667	1632	821	216	78	4 0
10	FLLLLKYRAREP	23	0	85	0	0	0 0
	LLLKYRAREPVTKAE	3033	3124	2857	2532	2028	639 169
	LLKYRAREPVTKAE	3078	3186	2918	2012	1419	303 40
	LKYRAREPVTKAE	2755	2544	1324	386	79	0 0
	KYRAREPVTKAE	50	0	0	0	0	0 0

15

TABLE VI

SEQ ID NO	PEPTIDE SEQUENCE	SEQ ID NO	PEPTIDE SEQUENCE
24	VAELVHFLLKYNARE	33	FLLKYNAREPVTK
25	VAELVHFLLKYNAR	34	FLLKYNAREPVT
26	VAELVHFLLKYRA	35	FLLKYNAREPV
27	AELVHFLLKYNARE	36	FLLKYNAREP
28	AELVHFLLKYNAR	37	LLKYNAREPVTKAE
29	AELVHFLLKYRA	38	LLKYNAREPVTKAE
30	ELVHFLLKYNARE	39	LKYNAREPVTKAE
31	ELVHFLLKYNAR	40	KYNAREPVTKAE
32	FLLKYNAREPVTKA		

**Example 9: Recognition of other MAGE proteins by clones 2 and 22**

The peptide MAGE-A3<sub>119-134</sub> (FLLKYNAREPVTKAE; SEQ ID NO:23) is present identically in the amino acid sequences of MAGE-A1 (amino acids 112-127), MAGE-A2 (amino acids 119-134) and MAGE-A6 (amino acids 119-134). Clones 2 and 22, but not clone 37, were stimulated by autologous EBV-B cells loaded with a MAGE-A1 recombinant protein according to the assays described above. Peptides recognized by clone 37 are also encoded by other MAGE proteins: SEQ ID NO:28 exists in MAGE-A12 and SEQ ID NOS:11 and 41 exist also in MAGE-A2, MAGE-A6. Thus, the two MAGE-A3.DR13 epitopes may be useful for clinical trials with other MAGE products.

Homologous peptide sequences are found in other cancer antigens including MAGE-A4, MAGE-A6, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A12, MAGE-B2, MAGE-C1. To determine if the CD4<sup>+</sup> T cell clones recognize these additional cancer associated antigens, the recombinant proteins, or synthesized peptides corresponding to the homologous region in these proteins, are used to load antigen presenting cells (such as EBV-B cells) to test for recognition by clones 2, 22 or 37 according to the assays described above. Homologous peptide which are recognized by clones 2, 22 or 37 may be regarded as functional variants of the MAGE-A3 peptides described herein.

**Example 10: identification of a MAGE-A3 peptide presented by HLA-DP**

**molecules****I. Cell lines, cytokines**

As described above.

5

**II. Production of recombinant MAGE-A3 proteins.****A. Production of His-MAGE-A3 protein in *E. coli***

Recombinant His-MAGE-A3 protein was produced as described above. The recombinant protein produced in *E. coli* (batch 97A21/1a) is hereafter referred to as protein MAGE-A3 (bacteria).

10

**B. Production of His-MAGE-A3 protein in Sf9 insect cells.**

Another recombinant MAGE-A3 protein was produced in our laboratory in *Spodoptera frugiperda* (Sf9) insect cells using a baculovirus expression system (PharMingen, San Diego, CA, USA). The coding sequence of MAGE-A3 was cloned in a baculovirus transfer vector (pAcGP67-A; PharMingen) downstream to the signal sequence of the gp67 surface protein of *Autographa californica* nuclear polyhedrosis virus (AcNPV), a strain of baculovirus. For easier purification, a sequence encoding a histidine tail was added at the C-terminus of the sequence of MAGE-A3. This construct allowed for secretion by the insect cells of a MAGE-A3 protein, hereafter referred to as MAGE-A3 (insect). DNA of the recombinant plasmid was co-transfected with DNA of lethally mutated AcNPV into Sf9 insect cells. Co-transfection allows recombination between homologous regions of the plasmid and the virus, transferring the foreign gene from the vector to the AcNPV DNA. Purification of the protein contained in the supernatant of Sf9 insect cell cultures involved the steps of filtration, anion exchange resin DEAE-sephadex chromatography, chromatography on a HighTrap chelating column saturated with NiCl<sub>2</sub>, affinity chromatography with an immobilized anti-MAGE-monoclonal antibody (mAb 57b, received from Dr. Giulio Spagnoli, Department Chirurgie, Kantonsspital, CH-4031 Basel, Switzerland), concentration and dialysis.

30

**III. Construction of retrovirus.**

A recombinant retrovirus, pMFG-Ii80-MAGE-A3-(IRES)-tNGFR was constructed. The tNGFR gene fragment encoding the extracellular and transmembrane regions of the low

affinity receptor for nerve growth factor was amplified from the plasmid pUC19-tNGFR, kindly provided by Dr. C. Traversari. The PCT was carried out using the following primers: sense: 5'-CCCTCATGAGGGCAGGTGCCACCG-3' (SEQ ID NO:88) antisense: 5'-CCCAGATCTCTAGAGGATTCCCCTGTTCCAC-3' (SEQ ID NO:89)

5 This PCR introduces a *Bsp*H1 site at the start codon and a *Bgl*2 site downstream from the stop codon. A *Bam*H1 site near the 3' end was deleted. The PCR product was cloned in pCR2.1 and sequenced. The tNGFR gene fragment was isolated from this vector as a *Bsp*H1-*Not*1 (from the pCR2.1 polylinker) fragment.

The IRES sequence derived from the encephalomyocarditis virus was transferred from 10 pGEM-EMC2 (kindly provided by Dr. J.-C. Renauld, Catholic University of Louvain, Brussels, Belgium) into pBluescript. An *Eco*R1-*Nco*1 fragment from pBluescript-IRES was used for further construction.

Both DNA fragments were ligated together into pCR2.1 *Eco*R1-*Not*1. This three fragment ligation resulted in a plasmid named pCR2.1-IRES-tNGFR.

15 A *Bam*H1-*Bgl*2 fragment from pCR2.1-IRES-tNGFR that comprises the IRES-tNGFR sequence was ligated into pMFG-Ii80 opened up with *Bam*H1 (pMFG-Ii80 is another term for pMFG-Ii as described in Example 6). This results in pMFG-Ii80-IRES-tNGFR with a unique *Bam*H1 site at the 3' end of the Ii80 gene fragment.

A *Bgl*2-*Bgl*2 fragment that contains the entire MAGE-A3 cDNA with a 5' *Bgl*2 site in 20 frame with the *Bam*H1 site at the 3' boundary of Ii80 was isolated from pCR2.1 MAGE-3(*Bgl*2) and inserted into pMFG-Ii80-IRES-tNGFR opened with *Bam*H1. The resulting plasmid pMFG-Ii80-MAGE-A3-IRES-tNGFR allows the simultaneous expression of both the Ii80-MAGE-A3 fusion protein and the membrane inserted tNGFR.

High titer MAGE 3 encoding recombinant retrovirus stocks were generated by 25 introducing plasmid pMFG-Ii80-MAGE-A3-IRES-tNGFR into PhoenixAMPHO packaging cells by transfection, as described above. Retrovirus stocks were harvested and used for transduction as described above.

#### IV. Processing of human blood.

30 Blood was processed as described above with minor modifications. For preparation of autologous plasma, after the first centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway), the upper part of the supernatant (plasma diluted 1/3) was collected, centrifuged at

500g for 10 min to remove the platelets. The supernatant was incubated at 56°C for 30 min, and stored at -20°C.

#### V. Mixed lymphocyte/dendritic cell culture.

5 Dendritic cells ( $5 \times 10^5$  per 500 microliters) were incubated at 37°C, 5% CO<sub>2</sub>, for 18-20 h in RPMI 1640 medium supplemented with 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine (AAG), 100 U/ml penicillin and 100 µg/ml streptomycin, IL-4 (100 U/ml), GM-CSF (100 ng/ml) and TNF-α (5 ng/ml) in the presence of 10 microgram of the MAGE-A3 protein (insect). Cells were washed and added at  $10^4$  per round-bottomed  
10 microwell to  $10^5$  autologous CD4<sup>+</sup> lymphocytes in 200 microliters IMDM medium supplemented with AAG (hereafter referred to as complete IMDM medium) and 1% autologous plasma, in the presence of IL-6 (1,000 U/ml) and IL-12 (10 ng/ml) (Fig. 16). The CD4<sup>+</sup> lymphocytes were restimulated on days 7, 14, 21 and 30 with autologous dendritic cells freshly loaded with protein MAGE-A3 (insect) (see above), and were grown in complete  
15 Iscove's medium supplemented with 10% human serum, IL-2 (10 U/ml) and IL-7 (5 ng/ml). Microcultures containing proliferating CD4<sup>+</sup> T cells were assessed on day 36 for their capacity to produce IFN-γ when stimulated with autologous EBV-B cells loaded with protein MAGE-A3 (bacteria). The reason for using another protein was to avoid taking into account the CD4<sup>+</sup> T cells reacting against contaminants contained in the batch of the protein used for loading the  
20 dendritic stimulator cells. Protein-pulsed EBV-B cells were washed and distributed at ~10,000 cells per round-bottomed microwell together with ~4,000 CD4<sup>+</sup> T lymphocytes in 100 µl of complete IMDM medium supplemented with 10% human serum and IL-2 (25 U/ml). After 20 h, the supernatant was collected and its IFN-γ content was measured by ELISA using reagents from Medgenix Diagnostic-Biosource (Fleurus, Belgium).  
25 Microculture C3, which produced a high level of IFN-γ after stimulation with protein MAGE-A3 (bacteria), was cloned by limiting dilution (Fig. 17).

#### VI. A CD4<sup>+</sup> T cell clone directed against a MAGE-A3 antigen.

Autologous EBV-B cells loaded with protein MAGE-A3 (bacteria) (20 microgram/ml)  
30 were used as stimulator cells ( $6 \times 10^3$  to  $10 \times 10^3$  cells per microwell) for the first 2 stimulations. Purified PHA (0.2 µg/ml, HA 16, MUREX DIAGNOSTICS, Dartford, UK) was used for the next stimulations. Allogeneic LG2-EBV-B cells ( $5 \times 10^3$  to  $10 \times 10^4$  cells per

microwell) were added as feeder cells. CD4<sup>+</sup> T cell clones were restimulated once a week and grown in complete IMDM medium supplemented with 10% human serum, IL-2 (50 U/ml) and 0.5  $\mu$ g/ml purified PHA (HA 16, MUREX DIAGNOSTICS, Dartford, UK) or EBV-B cells loaded with protein MAGE-A3 (bacteria). Occasionally, IL-4 (5 U/ml), 1-methyl-DL-tryptophan (100  $\mu$ M, Sigma Chemical Co., St. Louis, MO), or MRA (mycoplasma removal agent, 500 ng/ml, ICN) were also added.

EBV-B cells from hemochromatosis patient LB 1981 (LB-1981-EBV) were incubated with 20  $\mu$ g/ml of ovalbumin (OVA) or MAGE-A3 protein (bacteria) for 20 h. Ten thousand cells were distributed in microwells to which an aliquot of microculture C3 (~4,000 effector cells) was added. Several clones were obtained from microculture C3, in particular CD4<sup>+</sup> clones LB1981-CD4 527/C3.22 (referred to hereafter as clone C3.22) and LB1981-CD4 527/C3.43 (referred to hereafter as clone C3.43). The clones C3.22 and C3.43 (~3,000 cells) were stimulated with LB-1981-EBV incubated with 20  $\mu$ g/ml of MAGE-A3 (~16,000 cells) or transduced with a retrovirus coding for a fusion protein Ii-MAGE-A3 (~20,000 cells). IFN- $\gamma$  production in the supernatant was estimated after overnight culture by ELISA. They both recognized autologous EBV-B cells loaded with protein MAGE-A3 (bacteria) or transduced with a retrovirus encoding a truncated invariant chain (Ii)-MAGE-A3 fusion protein (Fig. 17). These (most probably twin) clones recognized cells expressing Ii-MAGE-A3 endogenously and therefore they are directed against a MAGE-A3-derived antigen and not against a contaminant in the batch of protein.

#### **VI. CD4 clone C3.22 recognizes MAGE-A3 peptide TQHFVQENYLEY (SEQ ID NO:86) on HLA-DPB1\* 0401**

The antigenic peptides were identified as described above, except that the peptides were tested at a concentration of 1  $\mu$ g/ml and that the test was performed in 100  $\mu$ l. Peptides LGDPKLLTQHFVQEN (SEQ ID NO:82), KKLLTQHFVQENYLEY (SEQ ID NO:83), TQHFVQENYLEYRQVP (SEQ ID NO:84) and VQENYLEYRQVPGSDP (SEQ ID NO:85) were tested. Autologous EBV-B cells were incubated for 1 h with 1  $\mu$ g/ml of each of the peptides. Clone C3.43 (3,000 cells) was then cocultured with 20,000 peptide-pulsed cells for 20 h. IFN- $\gamma$  production in the supernatant was measured by ELISA. Two peptides scored positive with clone C3.43, namely KKLLTQHFVQENYLEY (aa 243-258; SEQ ID NO:83 and TQHFVQENYLEYRQVP (aa 247-262; SEQ ID NO:84) (Fig. 18A). Another set of 12

amino acid peptides whose sequences were included in those of the two positive 16 amino acid peptides was tested. Clone C3.22 (4,000 cells) was cocultured with 10,000 peptide-pulsed cells for 20 h. Peptide TQHFVQENYLEY (aa 247-258; SEQ ID NO: 86) was recognized by clone C3.22. A significant recognition was already obtained at approximately 10 mM (Fig. 18B).

The recognition by clone C3.22 of cells loaded with peptide KKLLTQHFVQENYLEY (aa 243-258; SEQ ID NO:83) was abolished by an anti-HLA-DP antibody. Autologous EBV-B cells (20,000 cells) were incubated for 1 h with 1  $\mu$ g/ml of peptide KKLLTQHFVQENYLEY (SEQ ID NO:83), and washed. The peptide pulsed cells were then incubated for 24 h at 37°C under 8% CO<sub>2</sub> with clone C3.22 (3,000 cells) in the continuous presence (or not) of monoclonal antibodies 2B6 (against HLA-DR; 1/20), B7.21 (against HLA-DP; 1/100) or SPVL3 (against HLA-DQ; 1/100) (provided by Dr. A. Mulder, Bloodbank, Leiden). IFN- $\gamma$  production in the supernatant was measured by ELISA. Monoclonal antibody B7.21 abolished the recognition whereas the recognition was unchanged in the presence of monoclonal antibodies 2B6, or SPVL3 (Fig. 19) indicating that the presenting molecule was an HLA-DP molecule.

Patient LB1981 was typed DPB1\*0401. To confirm that DPB1\*0401 was the presenting HLA-DP molecule, additional EBV-B cell lines were tested. Autologous LB 1981-EBV or allogeneic-EBV-B cells were incubated for 1 h with 1  $\mu$ g/ml of peptides, and washed. CD4 clones (3,000 cells) were then incubated for 20 h with 20,000 peptide-pulsed EBV-B cells. IFN- $\gamma$  released in the supernatant was measured by ELISA. Those EBV-B cell lines expressing DPB1\*0401 were able to present the MAGE-A3<sub>243-258</sub> peptide to clone C3.22 (Table VII). DPB1\*0401 is expressed in approximately 64% of Caucasians.

Table VII. HLA-DPB1\*0401 cells present the two peptides MAGE-A3<sub>243-258</sub> and MAGE-A3<sub>247-258</sub> to CD4<sup>+</sup> clones C3.22 and C3.43

EBV-B cell line	HLA DPB1*	IFN-γ production (pg/ml) by clone C3.22	
		stimulated with peptide	
		MAGE-A3 <sub>243-258</sub>	MAGE-A3 <sub>247-258</sub>
<b>DPB1*0401 positive</b>			
LB 1981		> 4000	> 4000
LB 1622	DPB1*1301	> 4000	ND
LB 1118		> 4000	ND
LB 650		> 4000	> 4000
LB 1841	DPB1*0501	ND	> 4000
<b>DPB1*0401 negative</b>			
LB 1532	DPB1*0101	2	128
LB 1857	DPB1*0201/0301	0	80
<b>IFN-γ production (pg/ml) by clone C3.43</b>			
		stimulated with peptide	
		MAGE-A3 <sub>243-258</sub>	MAGE-A3 <sub>247-258</sub>
<b>DPB1*0401 positive</b>			
LB 1981		ND	> 4000
LB 1841	DPB1*0501	ND	> 4000
LB 650		ND	3314
LB 1801		> 4000	ND
<b>DPB1*0401 negative</b>			
LB 1532	DPB1*0101	ND	32
LB 1857	DPB1*0201/0301	ND	3
LB 1972	DPB1*0101	266	ND
ND = not determined			

## VII. Direct recognition of tumor cell lines

It was concluded above that the MAGE-A3 antigens recognized on HLA-DR13 cannot be presented at the surface of tumor cells, in accordance with the cytosolic or nucleolar localization of MAGE proteins. To determine if this was also the case with MAGE-A3 antigens presented by HLA-DPB1\*0401, HLA-DPB1\*0401 tumor cells expressing MAGE-A3 (stimulator cells; 20,000), pretreated or not with IFN-γ (100 U/ml) for 48 h, were cocultured for 20 h with 3,000 T cells of clones C3.22 or C3.43. IFN-γ released in the

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supernatant was measured by ELISA. As shown in Fig. 20, the MAGE-A3 epitope presented by HLA-DPB1\*0401 is recognized at the surface of some DPB1\*0401 tumor cell lines expressing MAGE-A3 (Fig. 20).

Clone C3.22 was also tested for its lytic activity. Target cells were  $^{51}\text{Cr}$ -labeled for 1  
5 hour and incubated with CD4 clone C3.22 at various effector-to-target ratios. Chromium  
release was measured after 4 hours. MEL stands for melanoma. Autologous EBV-B cells  
were also transduced with a retrovirus coding for a fusion protein Ii-MAGE-A3. Clone C3.22  
lysed both autologous EBV-B cells transduced with a retrovirus coding for a fusion protein Ii-  
MAGE-A3 and a HLA-DP4 melanoma cell line expressing MAGE-A3 (LB1622-MEL) (Fig.  
10 21).

Other aspects of the invention will be clear to the skilled artisan and need not be  
repeated here. Each reference cited herein is incorporated by reference in its entirety.

The terms and expressions which have been employed are used as terms of description  
15 and not of limitation, and there is no intention in the use of such terms and expressions of  
excluding any equivalents of the features shown and described or portions thereof, it being  
recognized that various modifications are possible within the scope of the invention.

We claim:

**CLAIMS**

1. An isolated MAGE-A3 HLA class II-binding peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42 and SEQ ID NO:86, or a functional variant thereof comprising one or more amino acid additions,  
5 substitutions or deletions.
2. The isolated HLA class II-binding peptide of claim 1 wherein the isolated peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30,  
10 SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:83 and SEQ ID NO:84.
3. The isolated HLA class II-binding peptide of claim 1 wherein the isolated peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:24,  
15 SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86.
- 20 4. The isolated HLA class II-binding peptide of claim 1, wherein the isolated peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:86.
5. The isolated HLA class II-binding peptide of claim 1, wherein the isolated peptide  
25 comprises an endosomal targeting signal.
6. The isolated HLA class II-binding peptide of claim 5, wherein the endosomal targeting signal comprises an endosomal targeting portion of human invariant chain Ii.
- 30 7. The isolated HLA class II-binding peptide of claim 1 wherein the isolated peptide is non-hydrolyzable.

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8. The isolated HLA class II-binding peptide of claim 7 wherein the isolated peptide is selected from the group consisting of peptides comprising D-amino acids, peptides comprising a -psi[CH<sub>2</sub>NH]-reduced amide peptide bond, peptides comprising a -psi[COCH<sub>2</sub>]-ketomethylene peptide bond, peptides comprising a -psi[CH(CN)NH]-(cyanomethylene)amino peptide bond, peptides comprising a -psi[CH<sub>2</sub>CH(OH)]-hydroxyethylene peptide bond, peptides comprising a -psi[CH<sub>2</sub>O]-peptide bond, and peptides comprising a -psi[CH<sub>2</sub>S]-thiomethylene peptide bond.
9. A composition comprising an isolated MAGE-A3 HLA class I-binding peptide and an isolated MAGE-A3 HLA class II-binding peptide, wherein the isolated MAGE-A3 HLA class II-binding peptide comprises the amino acid sequence selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42 and SEQ ID NO:86, or a functional variant thereof.
10. The composition of claim 9, wherein the MAGE-A3 HLA class I-binding peptide and the MAGE-A3 HLA class II-binding peptide are combined as a polytope polypeptide.
11. The composition of claim 9, wherein the isolated MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86.
12. The composition of claim 9, wherein the isolated MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:86.
13. The composition of claim 9, wherein the isolated MAGE-A3 HLA class II-binding peptide comprises an endosomal targeting signal.
14. The composition of claim 13, wherein the endosomal targeting signal comprises an endosomal targeting portion of human invariant chain Ii.

15. An isolated nucleic acid encoding a peptide selected from the group consisting of the peptide of claim 1, the peptide of claim 2, the peptide of claim 3 and the peptide of claim 5.
- 5 16. The isolated nucleic acid of claim 15, wherein the nucleic acid comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:43, SEQ ID NO:44 and SEQ ID NO:87.
- 10 17. An expression vector comprising the isolated nucleic acid of claim 15 operably linked to a promoter.
18. The expression vector of claim 17 wherein the nucleic acid comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:43, SEQ ID NO:44 and SEQ ID NO:87.
- 15 19. The expression vector of claims 17 or 18 further comprising a nucleic acid which encodes an HLA-DRB1/13 molecule or an HLA-DPB1 molecule.
- 20 20. A host cell transfected or transformed with an expression vector selected from the group consisting of the expression vector of claim 17, the expression vector of claim 18, and the expression vector of claim 19.
- 25 21. A host cell transfected or transformed with an expression vector selected from the group of the expression vector of claim 17 and the expression vector of claim 18, and wherein the host cell expresses an HLA-DRB1/13 molecule or an HLA-DPB1 molecule.
22. A method for enriching selectively a population of T lymphocytes with CD4<sup>+</sup> T lymphocytes specific for a MAGE-A3 HLA class II-binding peptide comprising:  
contacting an isolated population of T lymphocytes with an agent presenting a  
30 complex of the MAGE-A3 HLA class II-binding peptide and an HLA class II molecule in an amount sufficient to selectively enrich the isolated population of T lymphocytes with the CD4<sup>+</sup> T lymphocytes, wherein the HLA class II molecule is an HLA-DRB1/13 molecule or an

HLA-DPB1 molecule and wherein the MAGE-A3 HLA class II-binding peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42 and SEQ ID NO:86.

5 23. The method of claim 22 wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83,  
10 SEQ ID NO:84 and SEQ ID NO:86.

24. The method of claim 22 wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:86.

15

25. The method of claim 22, wherein the MAGE-A3 HLA class II binding peptide comprises an endosomal targeting portion of human invariant chain Ii.

26. A method for diagnosing a disorder characterized by expression of MAGE-A3  
20 comprising:

contacting a biological sample isolated from a subject with an agent that is specific for the MAGE-A3 HLA class II binding peptide, and

determining the interaction between the agent and the MAGE-A3 HLA class II binding peptide as a determination of the disorder, wherein the MAGE-A3 HLA class II-  
25 binding peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42 and SEQ ID NO:86, or a functional variant thereof.

27. The method of claim 26, wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:24,  
30 SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID

NO:83, SEQ ID NO:84 and SEQ ID NO:86.

28. The method of claim 26, wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:23,  
5 SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:86.

29. A method for diagnosing a disorder characterized by expression of a MAGE-A3 HLA class II-binding peptide which forms a complex with an HLA class II molecule, comprising:  
contacting a biological sample isolated from a subject with an agent that binds the  
10 complex; and  
determining binding between the complex and the agent as a determination of the disorder,  
wherein the HLA class II molecule is an HLA-DRB1/13 molecule or an HLA-DPB1 molecule and the MAGE-A3 HLA class II-binding peptide comprises an amino acid sequence  
15 selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42 and SEQ ID NO:86, or a functional variant thereof.

30. The method of claim 29, wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:24,  
20 SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86.

25 31. The method of claim 29, wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:86.

32. A method for treating a subject having a disorder characterized by expression of  
30 MAGE-3, comprising:  
administering to the subject an amount of a MAGE-A3 HLA class II-binding peptide sufficient to ameliorate the disorder, wherein the MAGE-A3 HLA class II-binding peptide

comprises an amino acid sequence selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42 and SEQ ID NO:86, or a functional variant thereof.

33. The method of claim 32, wherein the MAGE-A3 HLA class II binding peptide  
5 comprises an endosomal targeting signal.

34. The method of claim 33, wherein the endosomal targeting signal comprises an endosomal targeting portion of human invariant chain Ii.

10 35. The method of claim 32, wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID  
15 NO:83, SEQ ID NO:84 and SEQ ID NO:86.

36. The method of claim 32, wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:86.

20

37. A method for treating a subject having a disorder characterized by expression of MAGE-A3, comprising:

administering to the subject an amount of a MAGE-A3 HLA class I-binding peptide and an amount of a MAGE-A3 HLA class II-binding peptide sufficient to ameliorate the  
25 disorder,

wherein the MAGE-A3 HLA class II-binding peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42 and SEQ ID NO:86, or a functional variant thereof.

30 38. The method of claim 37, wherein the MAGE-A3 HLA class I-binding peptide and the MAGE-A3 HLA class II-binding peptide are combined as a polytope polypeptide.

39. The method of claim 37, wherein the MAGE-A3 HLA class II binding peptide comprises an endosomal targeting signal.
40. The method of claim 39, wherein the endosomal targeting signal comprises an  
5 endosomal targeting portion of human invariant chain Ii.
41. The method of claim 37, wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID  
10 NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86.
42. The method of claim 37, wherein MAGE-A3 HLA class II-binding peptide consists of  
15 an amino acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:86.
43. A method for treating a subject having a disorder characterized by expression of MAGE-3, comprising:  
20 administering to the subject an amount of an agent which enriches selectively in the subject the presence of complexes of an HLA class II molecule and a MAGE-A3 HLA class II-binding peptide, sufficient to ameliorate the disorder,  
wherein the HLA class II molecule is an HLA-DRB1/13 molecule or an HLA-DPB1 molecule and the MAGE-A3 HLA class II-binding peptide comprises an amino acid sequence  
25 selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42 and SEQ ID NO:86, or a functional variant thereof.
44. The method of claim 43, wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:24,  
30 SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID

NO:83, SEQ ID NO:84 and SEQ ID NO:86.

45. The method of claim 43, wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:23,  
5 SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:86.

46. The method of claim 43, wherein the agent comprises a MAGE-A3 HLA class II binding peptide.

10 47. The method of claim 46, wherein the MAGE-A3 HLA class II binding peptide comprises an endosomal targeting signal.

48. The method of claim 47, wherein the endosomal targeting signal comprises an endosomal targeting portion of human invariant chain Ii.

15

49. A method for treating a subject having a disorder characterized by expression of MAGE-A3, comprising:

administering to the subject an amount of autologous CD4<sup>+</sup> T lymphocytes sufficient to ameliorate the disorder, wherein the CD4<sup>+</sup> T lymphocytes are specific for complexes of an  
20 HLA class II molecule and a MAGE-A3 HLA class II-binding peptide,

wherein the HLA class II molecule is an HLA-DRB1/13 molecule or an HLA-DPB1 molecule and the MAGE-A3 HLA class II-binding peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42 and SEQ ID NO:86, or a functional variant thereof.

25

50. The method of claim 49, wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35,  
30 SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86.

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51. The method of claim 49, wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:86.

5 52. A method for identifying functional variants of a MAGE-A3 HLA class II binding peptide, comprising

selecting a MAGE-A3 HLA class II binding peptide which comprises an amino acid sequence selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42 and SEQ ID NO:86, an HLA class II binding molecule which binds the MAGE-A3 HLA class II binding peptide, and a T cell which is stimulated by the MAGE-A3 HLA class II binding peptide  
10 presented by the HLA class II binding molecule;

mutating a first amino acid residue of the MAGE-A3 HLA class II binding peptide to prepare a variant peptide;

determining the binding of the variant peptide to HLA class II binding molecule and  
15 the stimulation of the T cell, wherein binding of the variant peptide to the HLA class II binding molecule and stimulation of the T cell by the variant peptide presented by the HLA class II binding molecule indicates that the variant peptide is a functional variant.

53. The method of claim 52, further comprising the step of comparing the stimulation of  
20 the T cell by the MAGE-A3 HLA class II binding peptide and the stimulation of the T cell by the functional variant as a determination of the effectiveness of the stimulation of the T cell by the functional variant.

54. An isolated polypeptide which binds selectively a polypeptide of claim 3, provided  
25 that the isolated polypeptide is not an HLA class II molecule.

55. The isolated polypeptide of claim 54, wherein the isolated polypeptide is an antibody.

56. The antibody of claim 55, wherein the antibody is a monoclonal antibody.

30

57. The isolated polypeptide of claim 54, wherein the isolated polypeptide is an antibody fragment selected from the group consisting of a Fab fragment, a F(ab)<sub>2</sub> fragment or a

fragment including a CDR3 region selective for a MAGE-A3 HLA class II-binding peptide.

58. An isolated CD4<sup>+</sup> T lymphocyte which selectively binds a complex of an HLA class II molecule and a MAGE-A3 HLA class II-binding peptide,

5 wherein the HLA class II molecule is an HLA-DRB1/13 molecule or an HLA-DPB1 molecule and wherein the MAGE-A3 HLA class II-binding peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42 and SEQ ID NO:86., or a functional variant thereof.

10 59. The isolated CD4<sup>+</sup> T lymphocyte of claim 58 wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, 15 SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86.

60. The isolated CD4<sup>+</sup> T lymphocyte of claim 58 wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:86.

20

61. An isolated antigen presenting cell which comprises a complex of an HLA class II molecule and a MAGE-A3 HLA class II-binding peptide,

25 wherein the HLA class II molecule is an HLA-DRB1/13 molecule or an HLA-DPB1 molecule and wherein the MAGE-A3 HLA class II-binding peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:41, SEQ ID NO:42 and SEQ ID NO:86.

62. The isolated antigen presenting cell of claim 61 wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ 30 ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:23, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41,

SEQ ID NO:83, SEQ ID NO:84 and SEQ ID NO:86.

63. The isolated antigen presenting cell of claim 61 wherein the MAGE-A3 HLA class II-binding peptide consists of an amino acid sequence selected from the group consisting of SEQ  
5 ID NO:23, SEQ ID NO:34, SEQ ID NO:37, SEQ ID NO:38 and SEQ ID NO:86.

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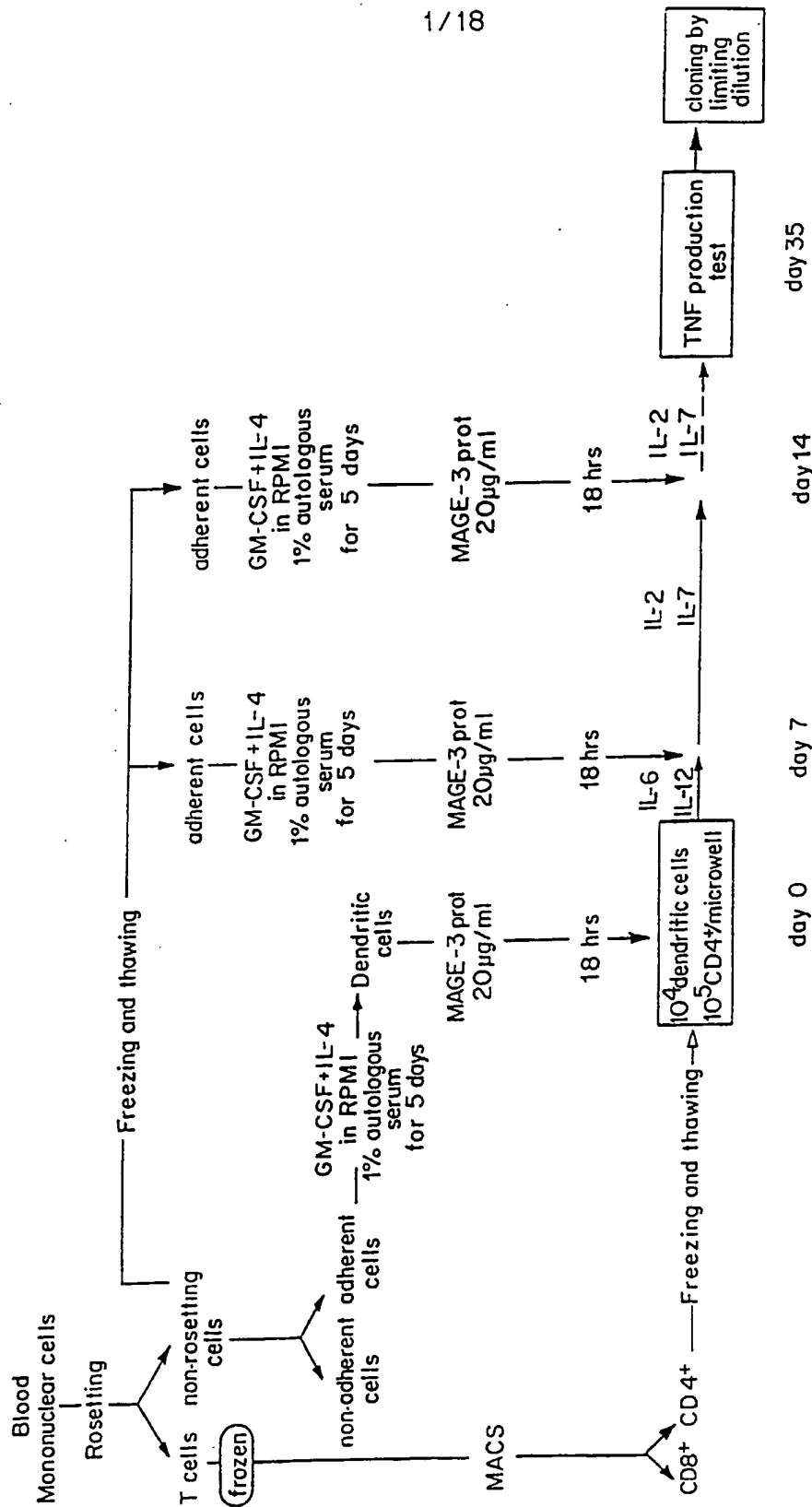


Fig. 1

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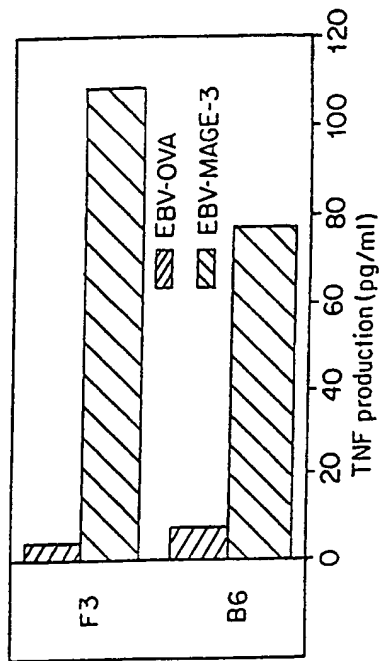


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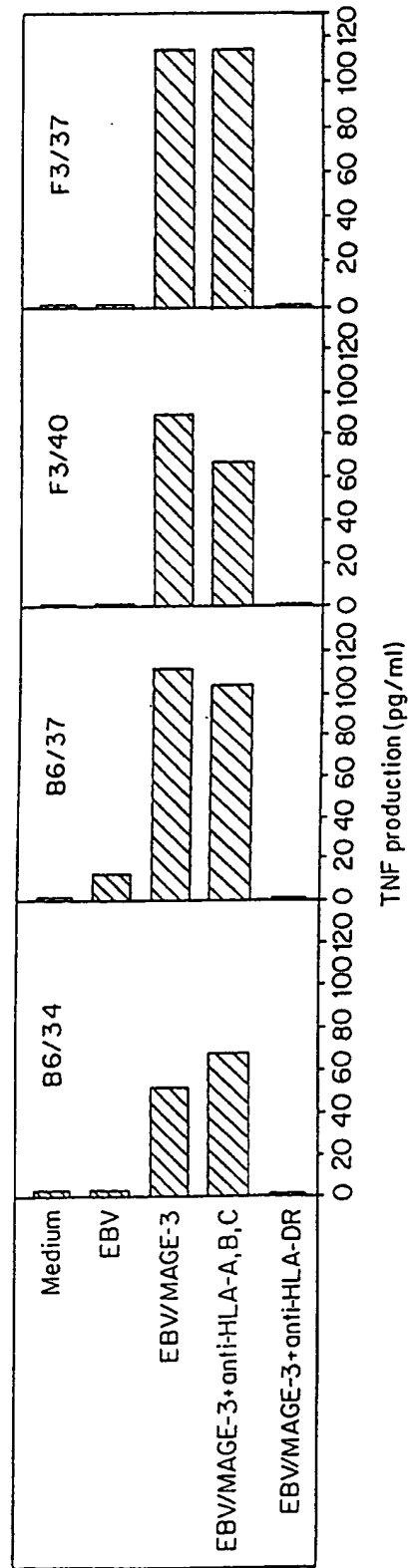
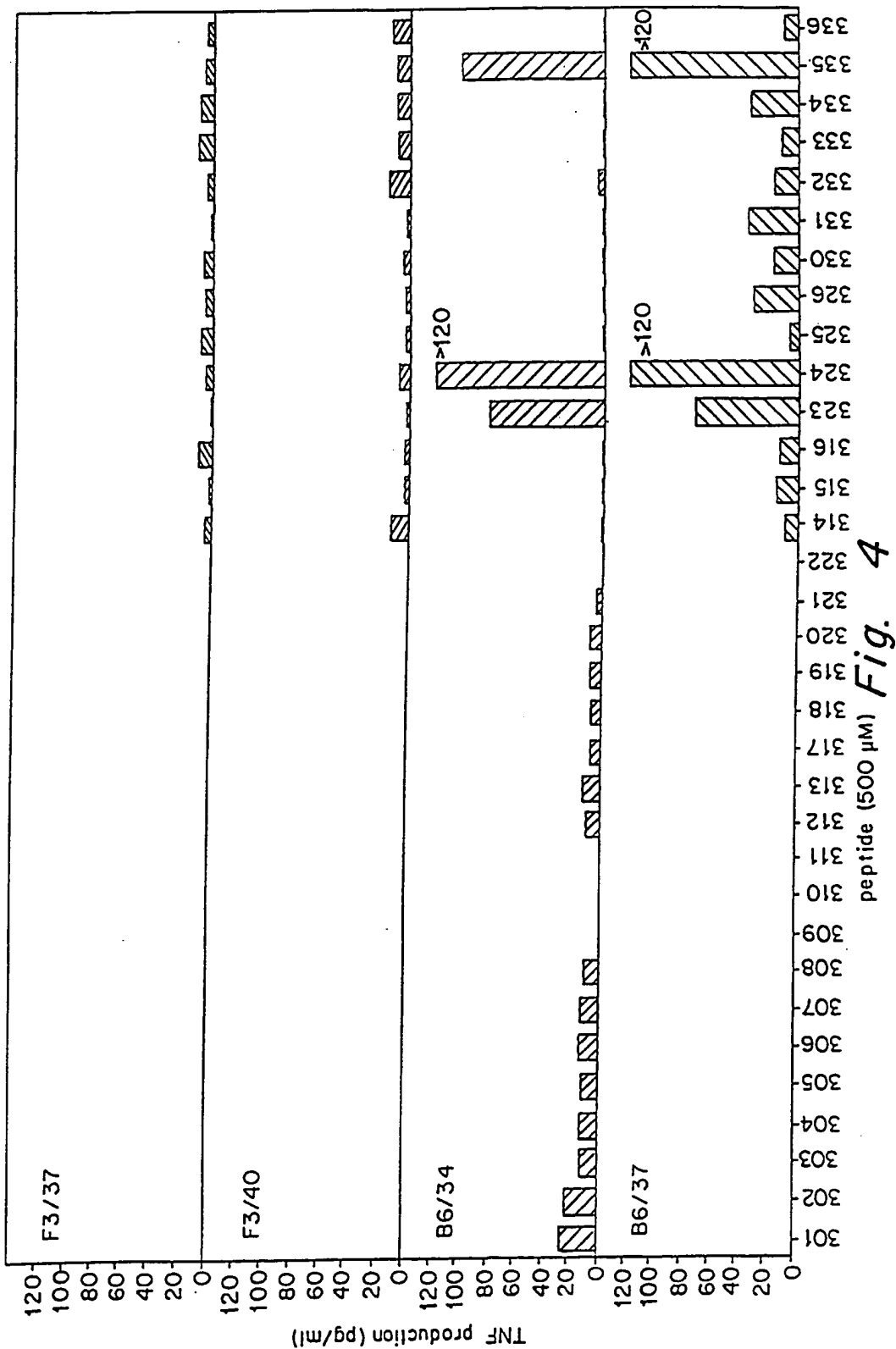


Fig. 3

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peptide (500  $\mu$ M) Fig. 4

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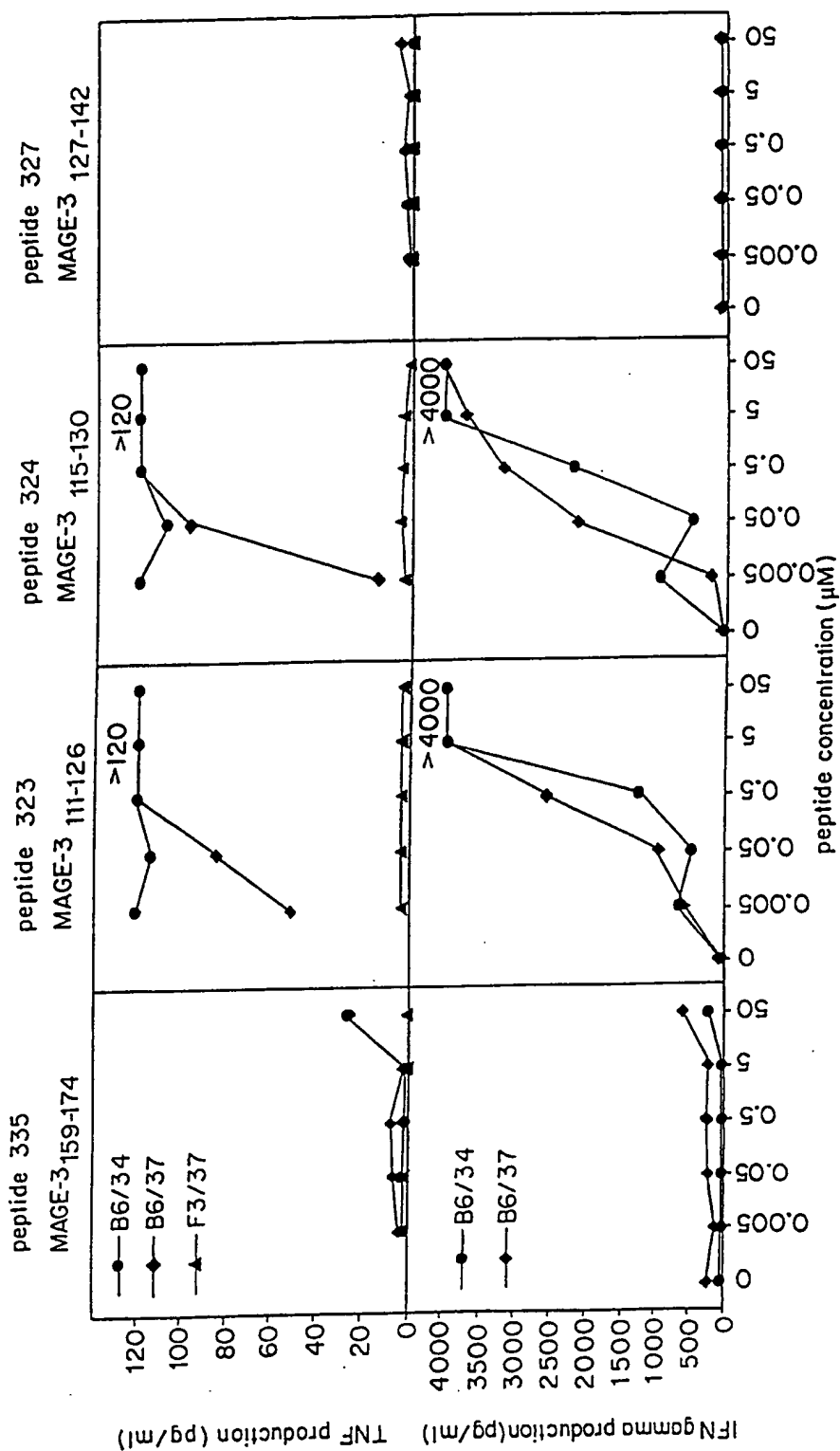
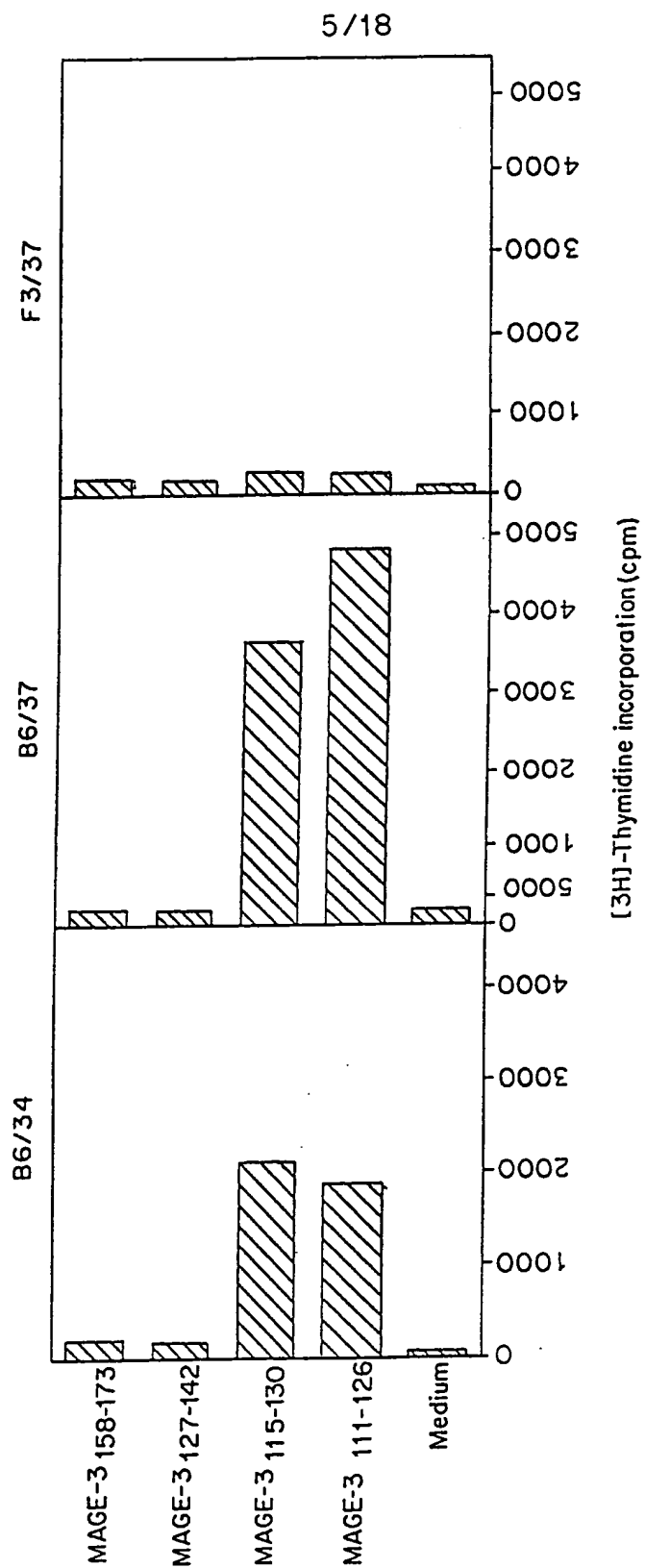


Fig. 5



*Fig. 6*

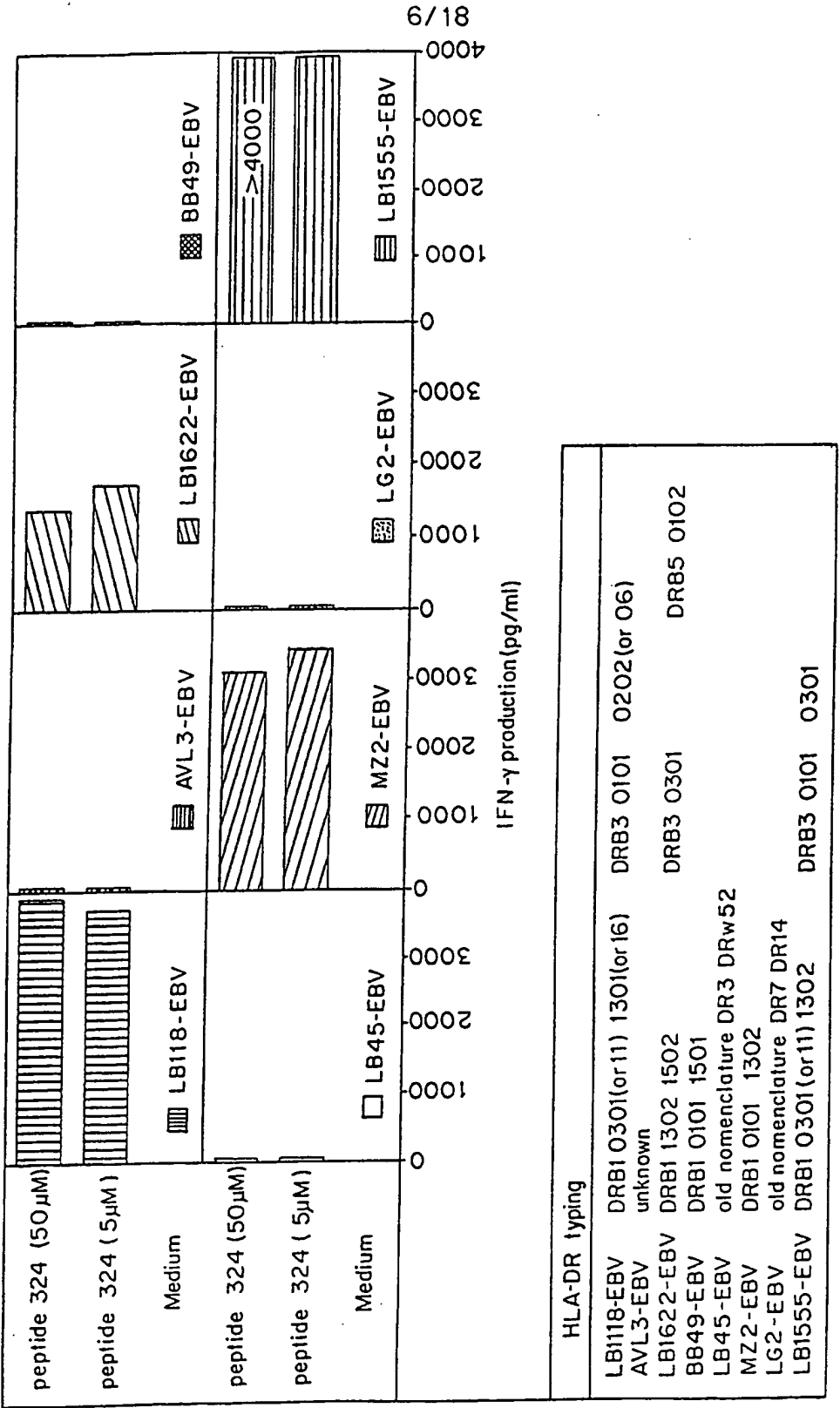
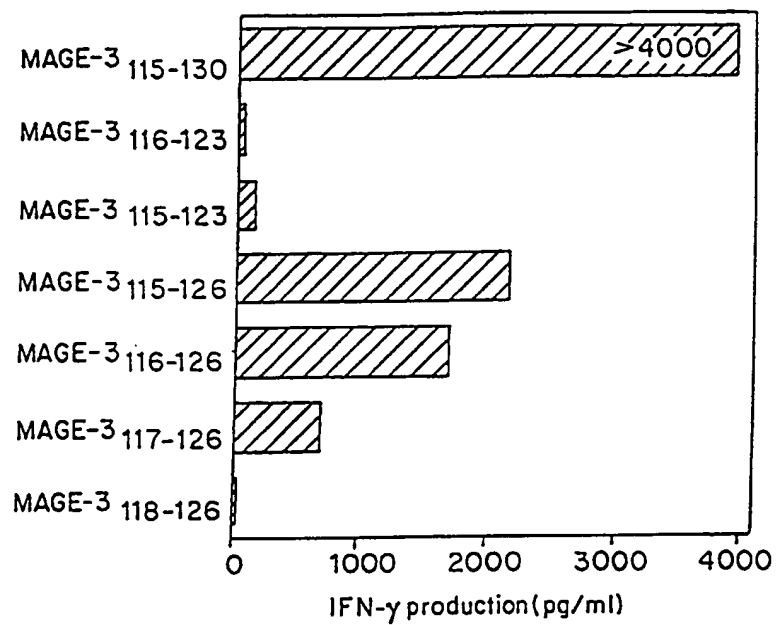


Fig. 7

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*Fig. 8*

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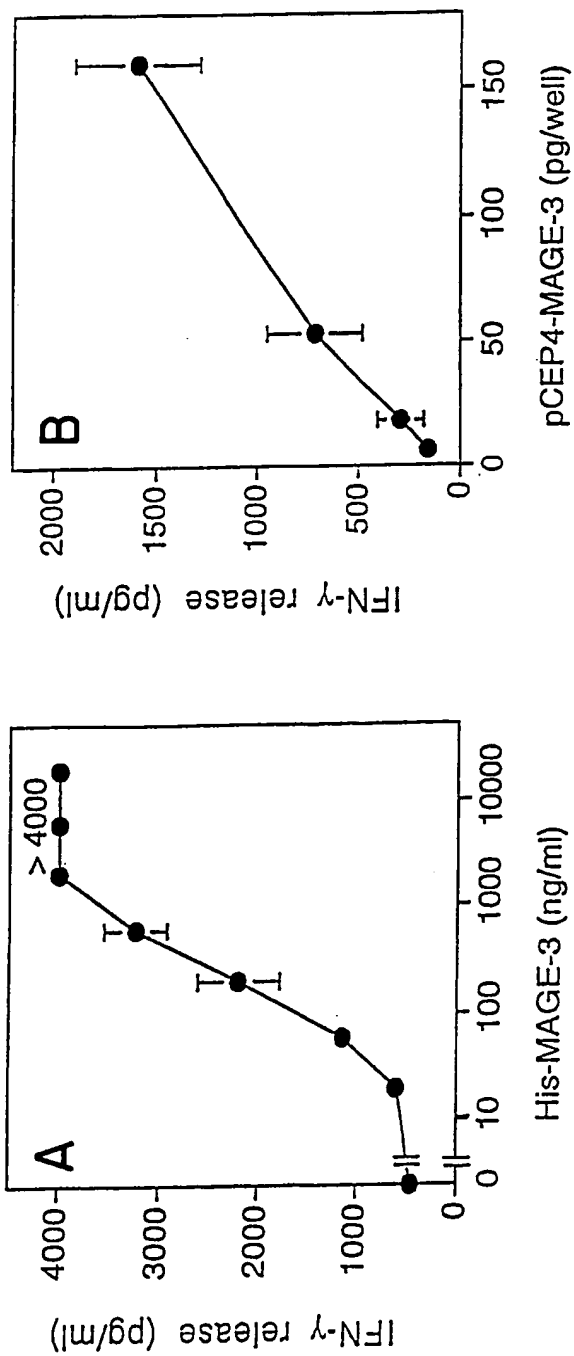
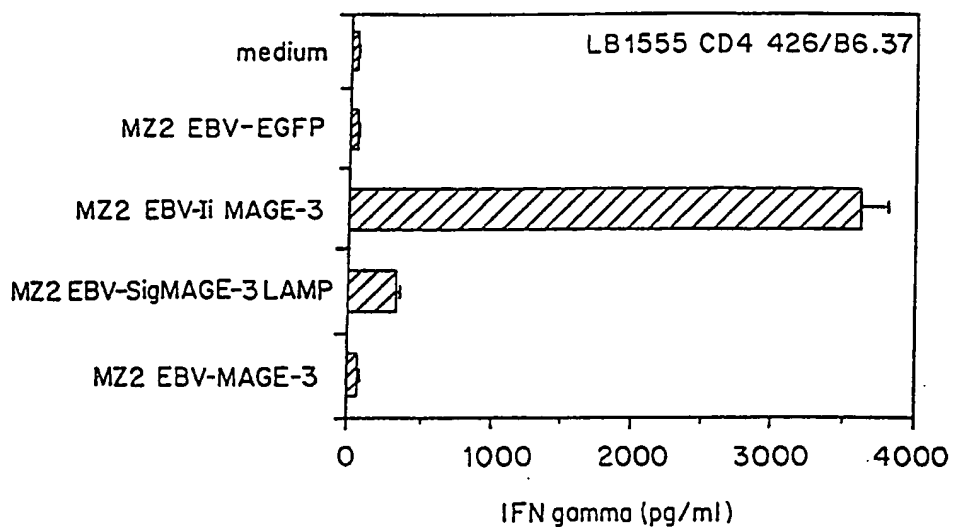
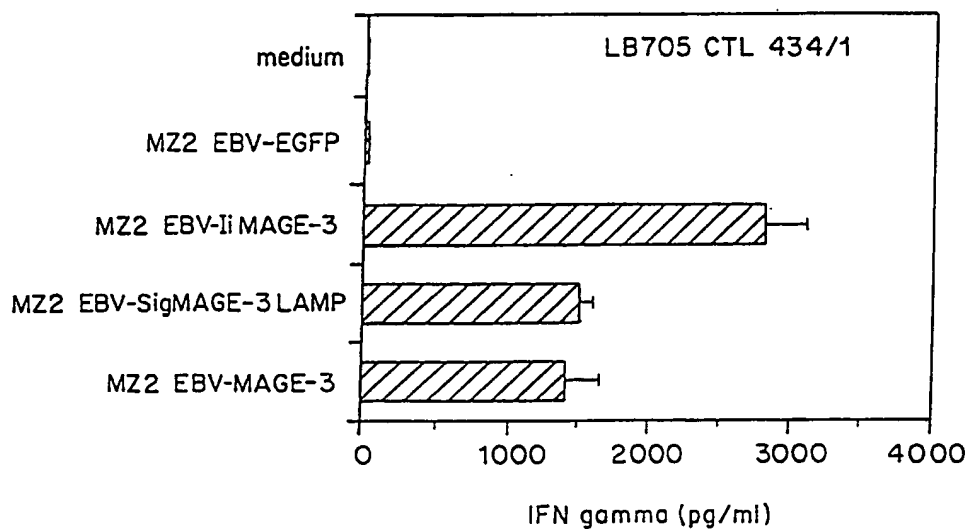


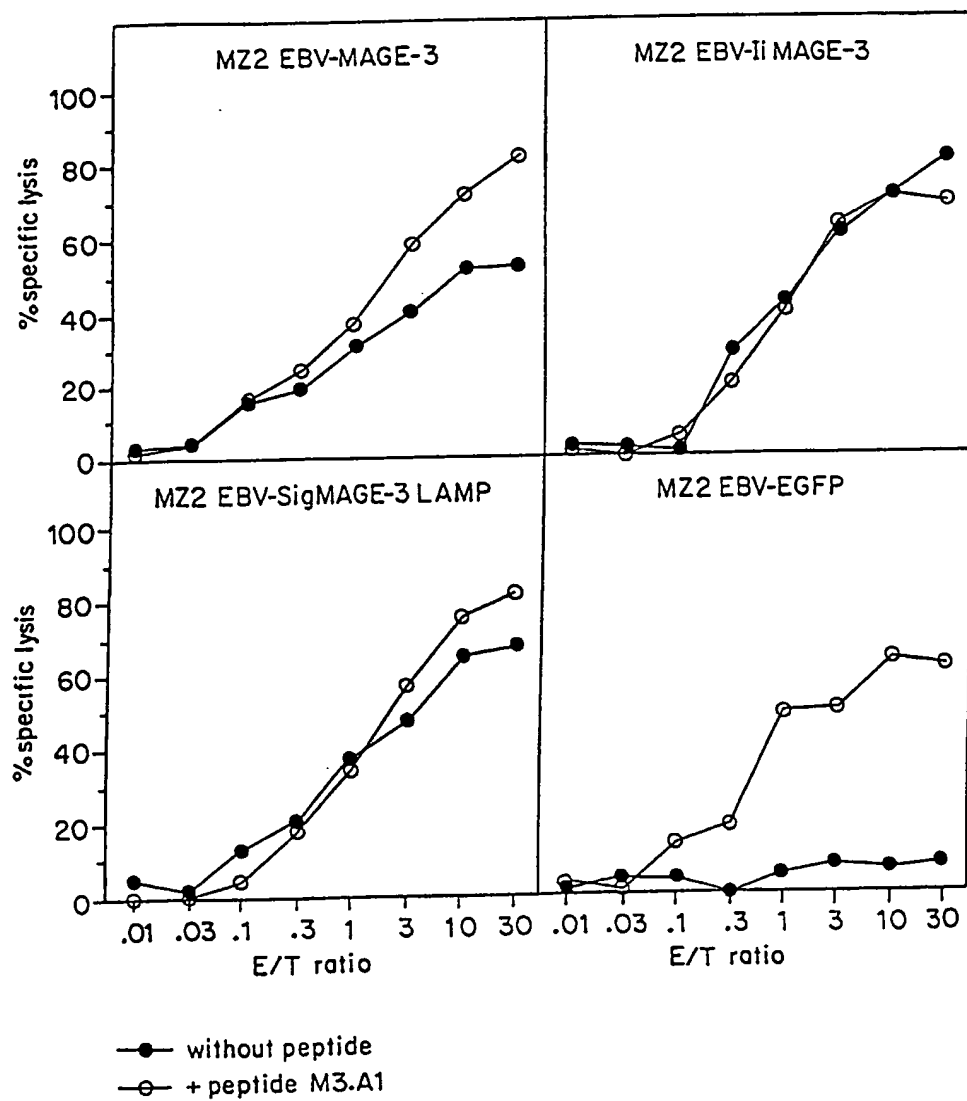
Fig. 9B

Fig. 9A

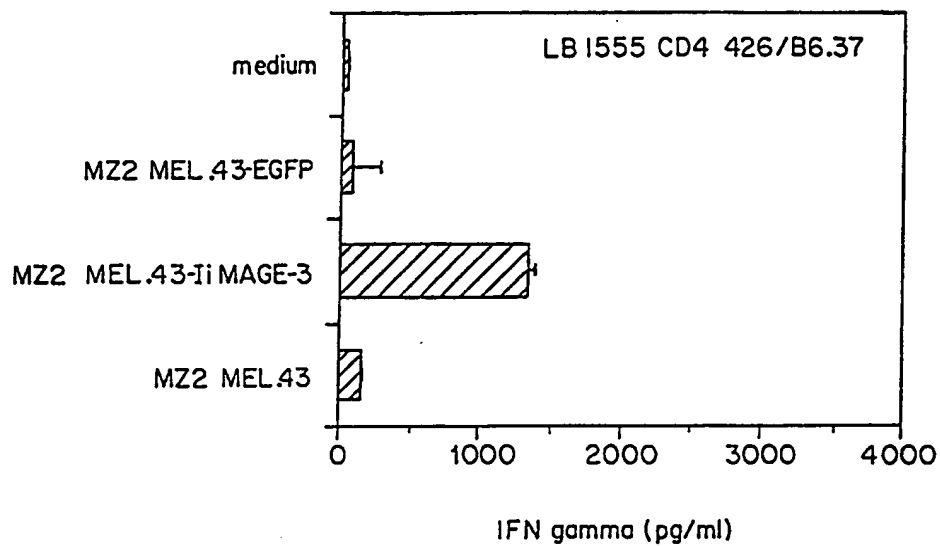
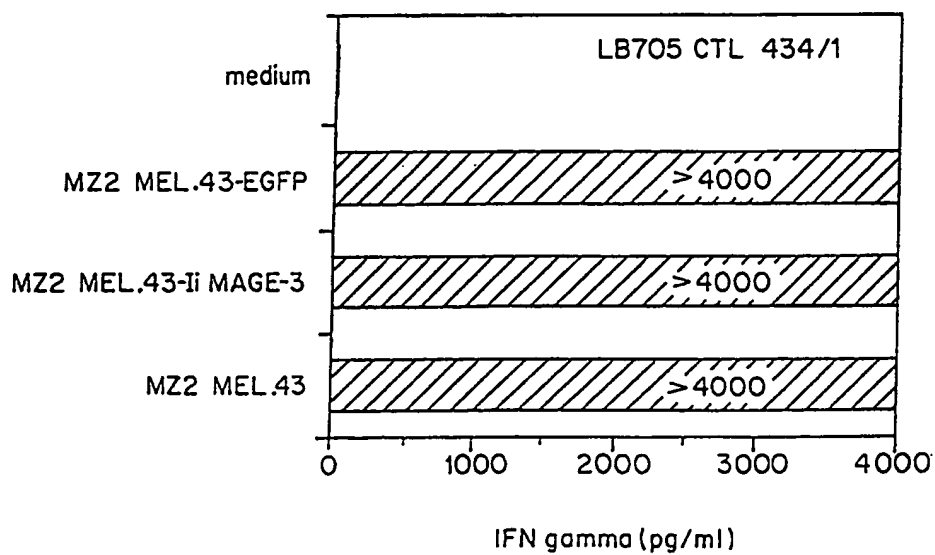
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*Fig. 10**Fig. 11*

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*Fig. 12*

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*Fig 13A**Fig 13B*

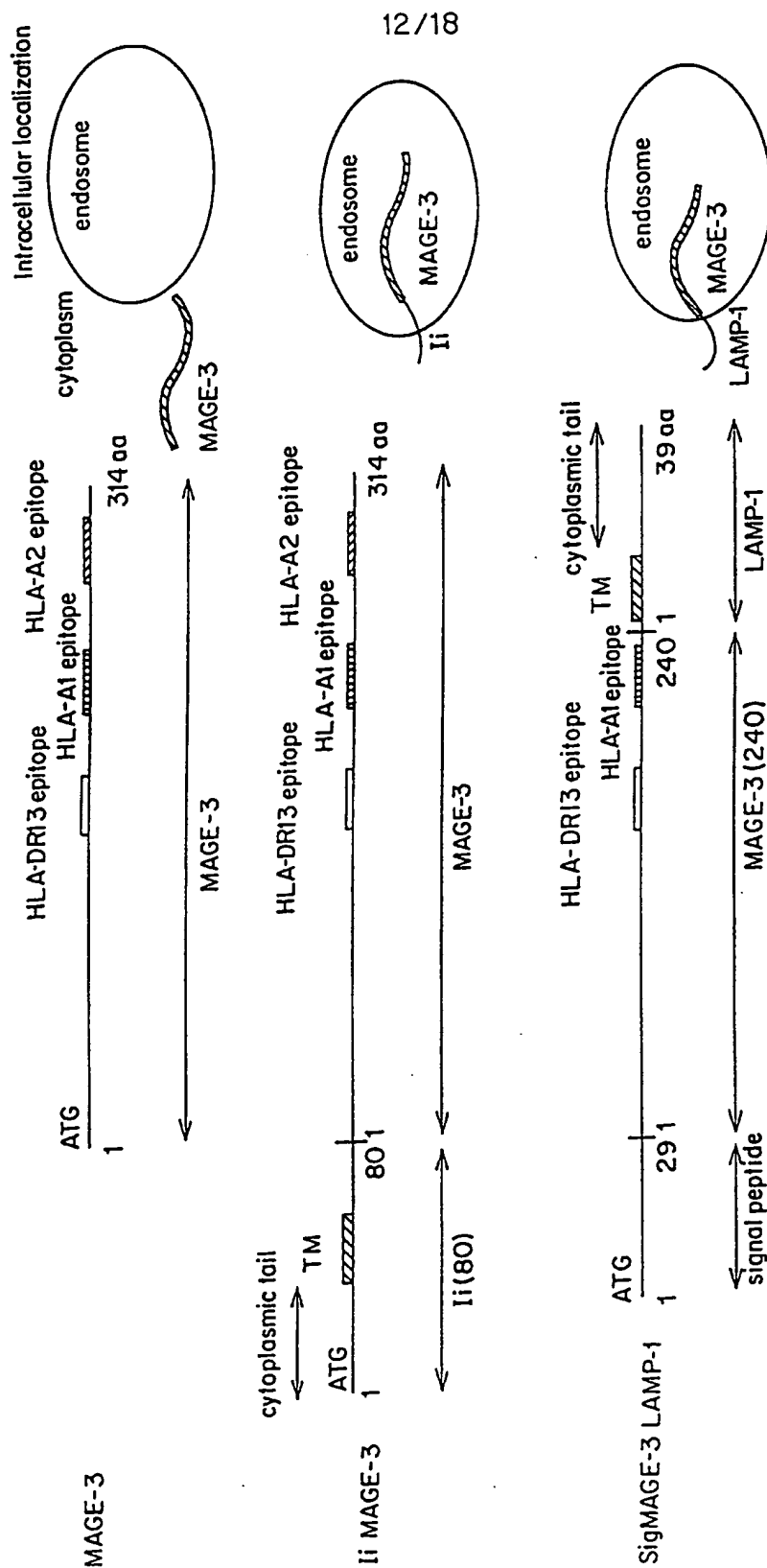
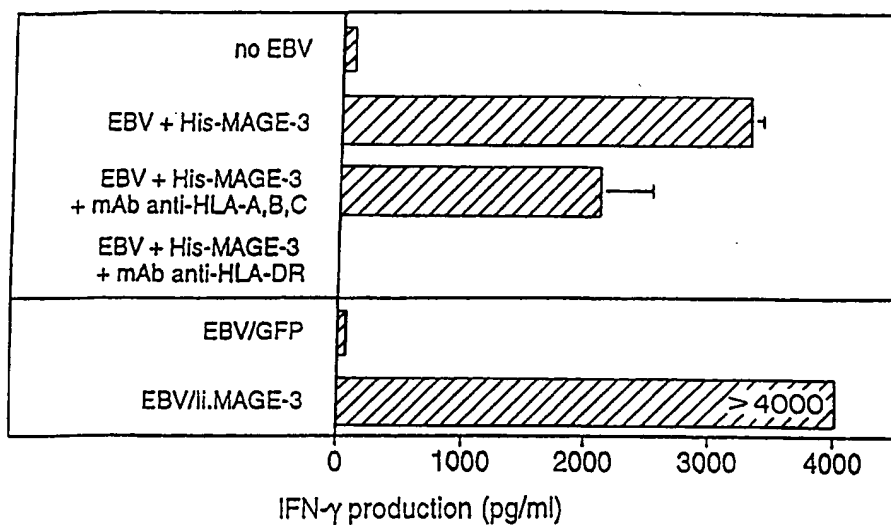
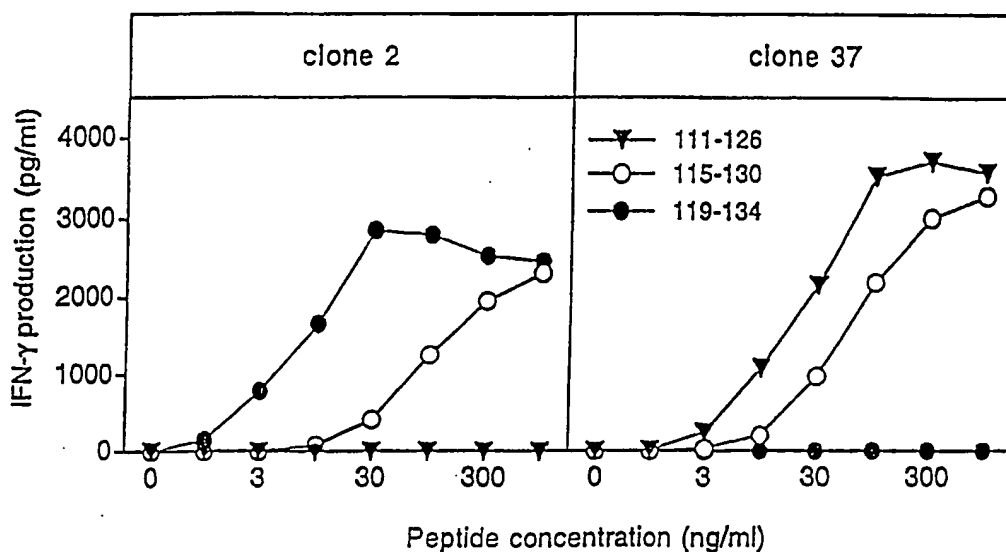
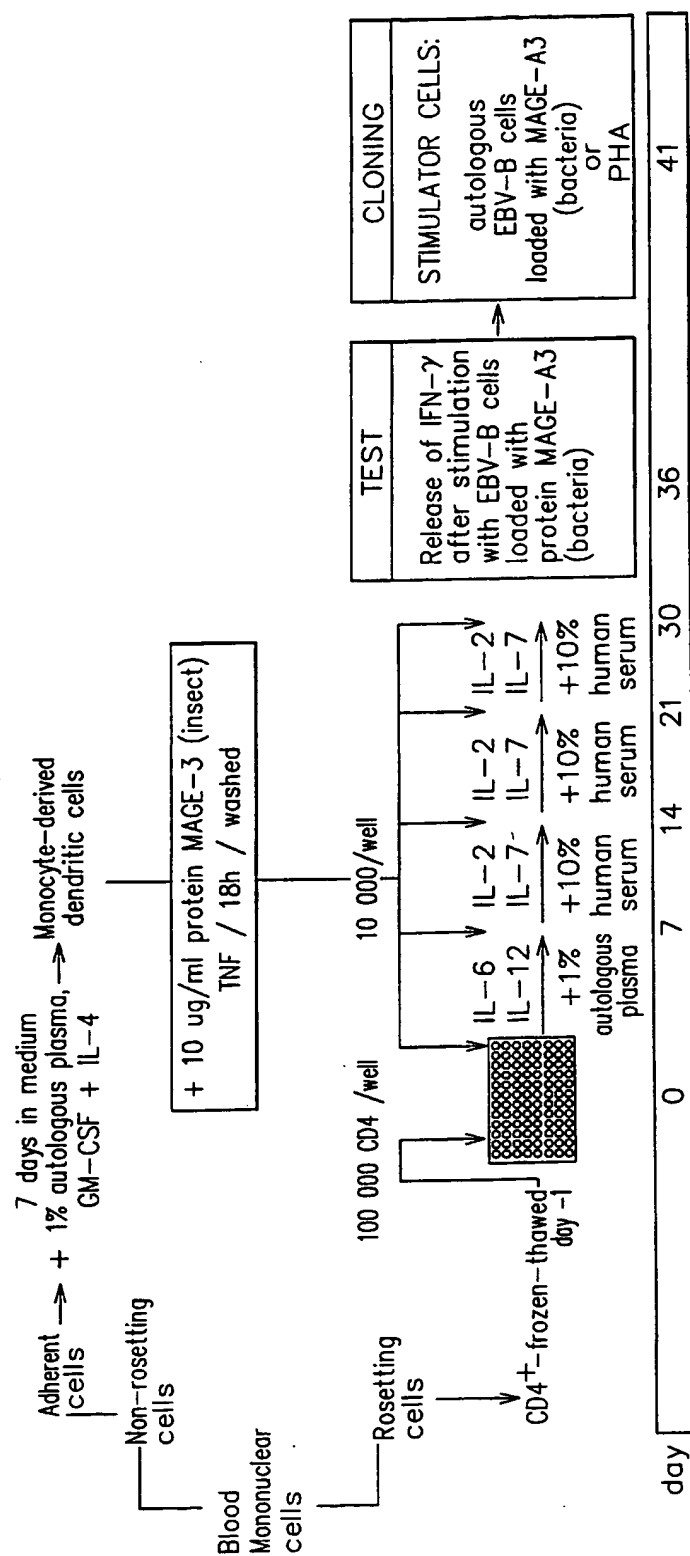


Fig. 14

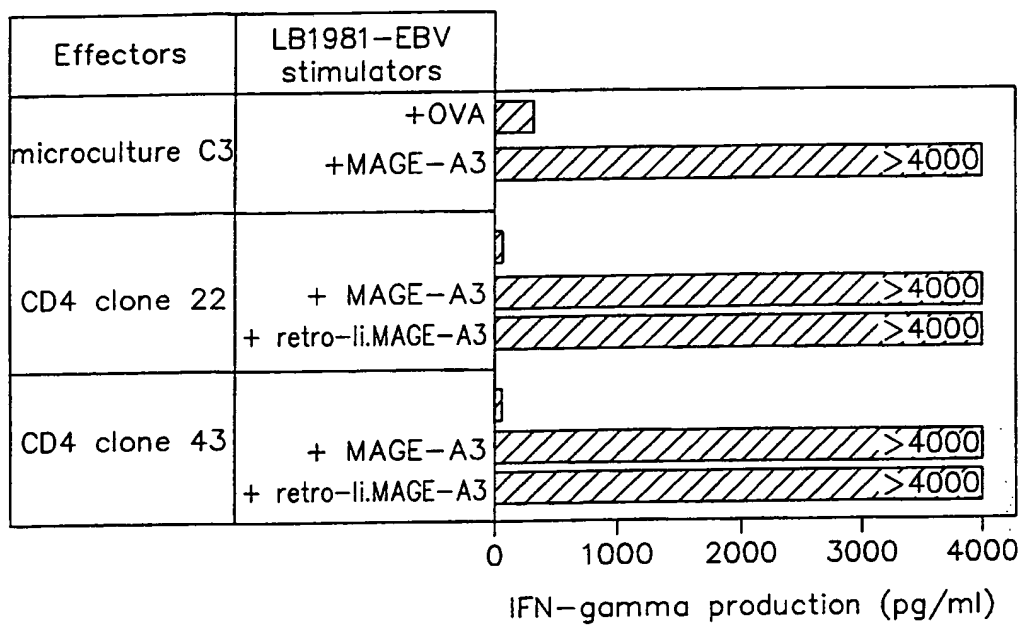
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*Fig. 15A**Fig. 15B*

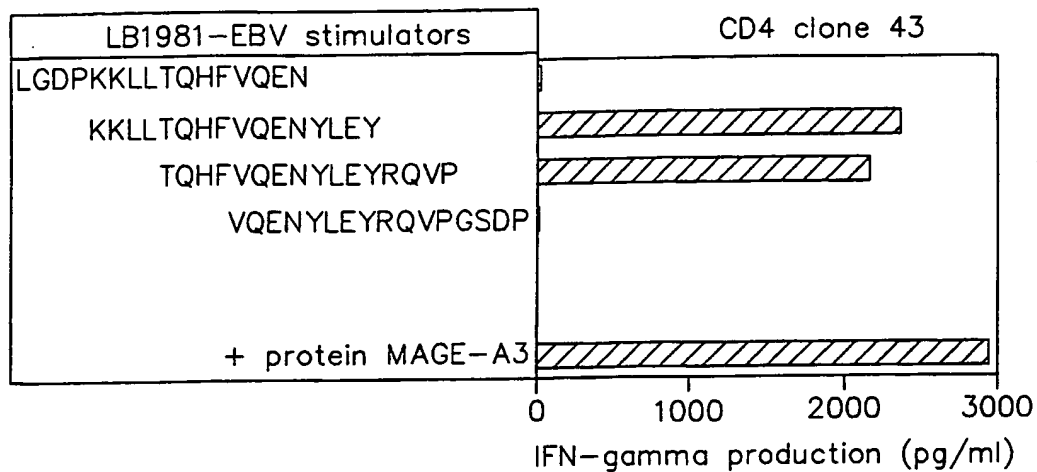
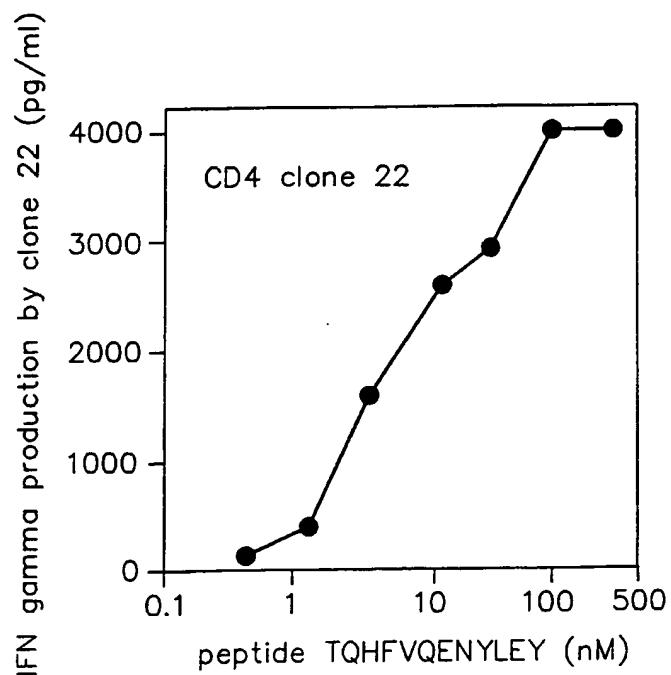


**Fig. 16**

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*Fig. 17*

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*Fig. 18A**Fig. 18B*

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Fig. 19

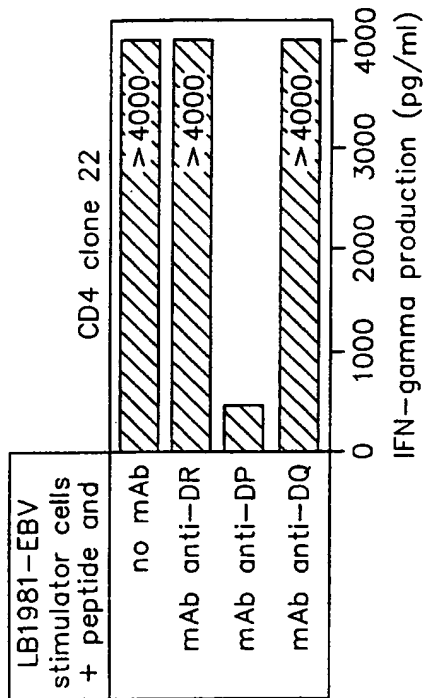
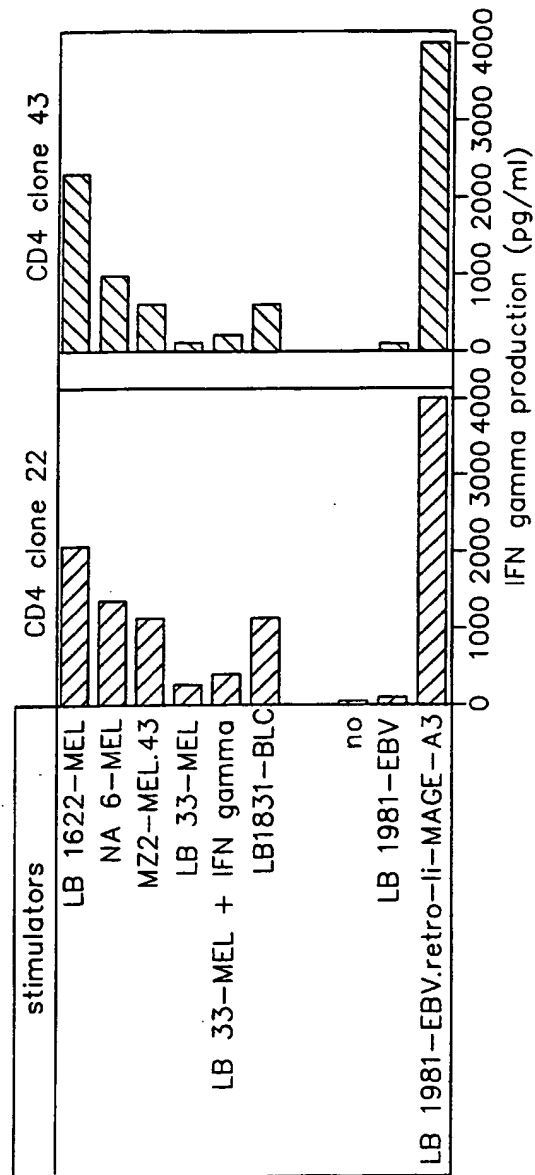


Fig. 20



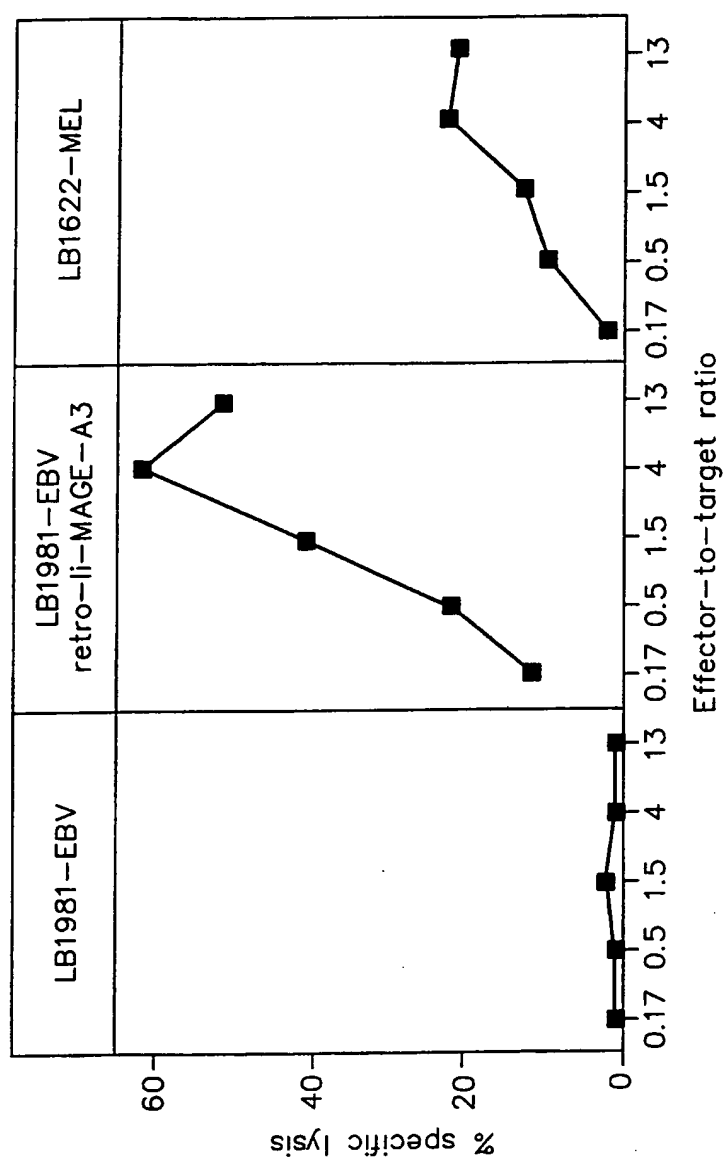


Fig. 21

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## SEQUENCE LISTING

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&lt;213&gt; Homo sapiens

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&lt;400&gt; 8

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/21230

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C07K14/47 G01N33/574 C07K16/30 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 23031 A (LUDWIG INST CANCER RES) 13 October 1994 (1994-10-13)  the whole document ---	1, 2, 9, 10, 13-18, 20
X	WO 98 04582 A (UNIV LEIDEN ; LUDWIG INST CANCER RES (US)) 5 February 1998 (1998-02-05) page 12, line 16 - line 17 Y page 36, line 1 - line 19 ---	1  5-8
X	WO 94 20127 A (CYTEL CORP) 15 September 1994 (1994-09-15) A page 112, line 13 ---	1 5-8
	--- -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

7 January 2000

Date of mailing of the international search report

14/01/2000

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Smalt, R

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/21230

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BRUGGEN VAN DER P ET AL: "A PEPTIDE ENCODED BY HUMAN GENE MAGE-3 AND PRESENTED BY HLA-A2 INDUCES CYTOLYTIC T LYMPHOCYTES THAT RECOGNIZE TUMOR CELLS EXPRESSING MAGE-3" EUROPEAN JOURNAL OF IMMUNOLOGY, DE, WEINHEIM, vol. 24, no. 12, page 3038-3043 XP000614539 ISSN: 0014-2980 page 3041, right-hand column, paragraph 2	61-63
Y	SANDERSON S ET AL: "EXPRESSION OF ENDOGENOUS PEPTIDE-MAJOR HISTOCOMPATIBILITY COMPLEX CLASS II COMPLEXES DERIVED FROM INVARIANT CHAIN-ANTIGEN FUSION PROTEINS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, US, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, vol. 92, no. 16, page 7217-7221 XP002025345 ISSN: 0027-8424 the whole document	5, 6
Y	SPATOLA A F: "Peptide backbone modifications: a structure-activity analysis of peptides containing amide bond surrogates" CHEMISTRY AND BIOCHEMISTRY OF AMINO ACIDS, PEPTIDES, AND PROTEINS, XX, XX, vol. 7, page 267-357 XP002086355 the whole document	7, 8
P, X	CHAUX, P. ET AL.: "Identification of MAGE-3 epitopes presented by HLA-DR molecules to CD4+ T lymphocytes." JOURNAL OF EXPERIMENTAL MEDICINE, vol. 189, no. 5, 1 March 1999 (1999-03-01), pages 767-77, XP000857617 the whole document	1-63
P, X	WO 99 14326 A (STROOBANT VINCENT ; HEIRMAN CARLO (BE); CHAUX PASCAL (BE); CORTHALS) 25 March 1999 (1999-03-25)  the whole document	1, 2, 5-10, 13-22, 25, 26, 29, 32-34, 37-40, 43, 47-49, 52-63

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/21230

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	<p>WO 99 45954 A (EPIMMUNE INC) 16 September 1999 (1999-09-16)</p> <p>the whole document -----</p>	<p>1-3, 9-11, 15-18, 20,22, 23,32, 35,37, 38,41</p>

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/ 21230

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 32-51  
are directed to a method of treatment of the human/animal  
body, the search has been carried out and based on the alleged  
effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such  
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all  
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment  
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report  
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is  
restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/21230

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